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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DOCKET NUMBER	ANTICIPATED CLASSIFICATION OF THIS APPLICATION:		PRIOR APPLICATION	
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30436.11US06	unknown	Unknown	P. Gambel	1644

CERTIFICATE UNDER 37 CFR 1.10:

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By: Richelle Ann Domingo
Name: Richelle Ann Domingo

DIVISIONAL APPLICATION UNDER 37 C.F.R. § 1.53

Assistant Commissioner for Patents
Washington, DC 20231

Dear Sir:

This is a request for filing a divisional application under 37 CFR § 1.53 of Serial No. 07/722,101, filed on June 27, 1991 entitled LIGAND FOR CD28 RECEPTOR ON B CELLS AND METHODS by the following inventor(s):

Full Name Of Inventor	Family Name Linsley	First Given Name Peter	Second Given Name S.
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1. ☒ Enclosed is a true and correct copy of the prior application; including the specification, claims, drawings, oath or declaration showing the applicant's signature, and any amendments referred to in the oath or declaration filed to complete the prior application. (It is noted that no amendments referred to in the oath or declaration filed to

complete the prior application introduced new matter therein.) The copy of the prior application is as follows: 78 pages of specification, 76 claims, 1 pages of abstract, 17 sheets of drawings, and 3 pages of oath or declaration.

2. ☒ Cancel in this application original claims 1-18 and 20-76 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)
3. ☒ The filing fee is calculated below:

CLAIMS AS FILED

NUMBER FILED	NUMBER EXTRA		RATE	FEE
TOTAL CLAIMS: 3 -20	0	x	\$0.00	\$0.00
INDEPENDENT CLAIMS 2 -3	0	x	\$0.00	\$0.00
			BASIC FILING FEE:	\$690.00
			TOTAL FILING FEE:	\$690.00

☐ A Verified Statement that this filing is by a small entity is already filed in the prior application.

☐ A Verified Statement that this filing is by a small entity is attached.

4. ☒ Payment of fees:
☒ Attached is a check in the amount of \$690.00.
☐ Please charge Deposit Account No. 50-0306.
5. ☒ The Commissioner is hereby authorized to charge any additional fees as set forth in 37 CFR §§ 1.16 to 1.18 which may be required by this paper or credit any overpayment to Account No. 50-0306.
6. ☒ Amend the specification by inserting before the first line the sentence:

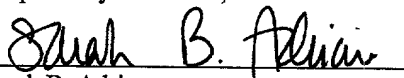
"This is a Divisional of application Serial No. 08/219,200, filed March 29, 1994, which is a file wrapper continuation of U.S. Serial No. 07/722,101, filed June 27, 1991, now abandoned, which was a continuation-in-part of U.S. Serial No. 547,980, filed July 2, 1990, now abandoned, which was a continuation-in-part of U.S. Serial No. 498,949, filed March 26, 1990, now abandoned, which application(s) are incorporated herein by reference."
7. ☐ A set of formal drawings (____ sheets) is enclosed.
8. ☐ Priority of application Serial No. _____, filed on _____ in _____, is claimed under 35 U.S.C. 119.
☐ The certified copy has been filed in prior application Serial No. _____, filed _____.
9. ☒ The prior application is assigned of record to Bristol-Myers Squibb Company.

10. ☒ The Power of Attorney in the prior application is to:

Mandel & Adriano
35 N. Arroyo Parkway, Suite 60
Pasadena, California 91103

11. ☒ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)
- ☐ Fee for excess claims is attached.
12. ☐ A petition and fee has been filed to extend the term in the prior application until _____. A copy of the petition for extension of time in the prior application is attached.
13. ☐ The inventor(s) in this application are less than those named in the prior application and it is requested that the following inventors identified above for the prior application be deleted:
14. ☐ Also Enclosed:
15. ☒ Address all future communications to the **Attention of Sarah B. Adriano** (may only be completed by attorney or agent of record) at the address below.
16. ☒ A return postcard is enclosed.

Respectfully submitted,



Sarah B. Adriano
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Peter S. Linsley et al.
Serial No. : Not yet known Examiner: P. Gambel
Filed : Herewith Group Art Unit: 1644
For : LIGAND FOR CD28 RECEPTOR ON B CELLS AND METHODS

35 N. Arroyo Parkway, Suite 60
Pasadena, California 91103
September 21, 2000

Assistant Commissioner of Patents
Washington, D.C. 20231

SIR:

**PRELIMINARY AMENDMENT SUBMITTED IN CONNECTION WITH THE
ACCOMPANYING DIVISIONAL APPLICATION
FILED UNDER 37 C.F.R. §1.53**

Please amend the subject application as follows:

In the Claims:

Please delete claims 1-18 and 20-76 without prejudice and add new claims 77-78 as follows.

- 77. (new) A method for blocking binding of B7 positive B cells to CD28 antigen to inhibit CD28 specific T cell interaction comprising contacting B7 positive B cells with a monoclonal antibody or fragment thereof which recognizes and binds B7 positive B cells in sufficient amounts effective to block binding of said CD28 antigen to said B7.—
- 78. (new) The method of claim 77, wherein said monoclonal antibody is mAb BB-1.

REMARKS

Support for claim 77 may be found in the originally filed claim 19-22.

Support for claim 78 may be found in the originally filed claim 19-22.

The subject application is a divisional application of U.S. Serial No. 07/722,101, filed June 27, 1991. The parent application, U.S. Serial No. 08/219,200, is pending, and the subject continuation application is co-pending therewith in fulfillment of the provisions of 35 U.S.C. §120.

Only claims 19 and 77-78 are pending. Applicants maintain that this amendment to the specification does not raise an issue of new matter and accordingly request that the amendments be entered.

No fee is deemed necessary in connection with the filing of this Amendment. If any fee is necessary, the Patent Office is authorized to charge any additional fee to Deposit Account No. 50-0306.

Respectfully submitted,



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LIGAND FOR CD28 RECEPTOR
ON B CELLS AND METHODS

BY

PETER S. LINSLEY, JEFFREY A. LEDBETTER,
NITIN K. DAMLE AND WILLIAM BRADY

007660 2929960

LIGAND FOR CD28 RECEPTOR ON B CELLS AND METHODS

by

Peter S. Linsley, Jeffrey A. Ledbetter,
Nitin K. Damle and William Brady

FIELD OF THE INVENTION

The present invention relates to the identification of an interaction between the CD28 receptor and its ligand, the B7 antigen, and to a method for regulating cellular interactions using the antigen, fragments and derivatives thereof.

BACKGROUND OF THE INVENTION

5 The generation of a T lymphocyte ("T cell") immune response is a complex process involving cell-cell interactions (Springer et al., A. Rev. Immunol. 5:223-252 (1987)), particularly between T and B cells, and production of soluble immune mediators (cytokines or lymphokines) (Dinarello and Mier, New Engl. Jour. Med. 317:940-945 (1987)). This response is regulated by several T-cell surface receptors, including
10 the T-cell receptor complex (Weiss et al., Ann. Rev. Immunol. 4:593-619 (1986)) and other "accessory" surface molecules (Springer et al., (1987) supra). Many of these accessory molecules are naturally occurring cell surface differentiation (CD) antigens defined by the reactivity of monoclonal
15 antibodies on the surface of cells (McMichael, Ed., Leukocyte Typing III, Oxford Univ. Press, Oxford, N.Y. (1987)).

20 One such accessory molecule is the CD28 antigen, a homodimeric glycoprotein of the immunoglobulin superfamily (Aruffo and Seed, Proc. Natl. Acad. Sci. 84:8573-8577 (1987)) found on most mature human T cells (Damle et al., J. Immunol. 131:2296-2300 (1983)). Current evidence suggests that this molecule functions in an alternative T cell activation pathway distinct from that initiated by the T-cell receptor complex

(June et al., Mol. Cell. Biol. 7:4472-4481 (1987)).

Monoclonal antibodies (mAbs) reactive with CD28 antigen can augment T cell responses initiated by various polyclonal stimuli (reviewed by June et al., supra). These stimulatory effects may result from mAb-induced cytokine production (Thompson et al., Proc. Natl. Acad. Sci 86:1333-1337 (1989); Lindsten et al., Science 244:339-343 (1989)) as a consequence of increased mRNA stabilization (Lindsten et al., (1989), supra). Anti-CD28 mAbs can also have inhibitory effects, i.e., they can block autologous mixed lymphocyte reactions (Damle et al., Proc. Natl. Acad. Sci. 78:5096-6001 (1981)) and activation of antigen-specific T cell clones (Lesslauer et al., Eur. J. Immunol. 16:1289-1296 (1986)).

The in vivo function of CD28 antigen is not known, although its structure (Aruffo and Seed, (1987), supra) suggests that like other members of the immunoglobulin superfamily (Williams and Barclay, Ann. Rev. Immunol. 6:381-405 (1988), it might function as a receptor. CD28 antigen could conceivably function as a cytokine receptor, although this seems unlikely since it shares no homology with other lymphokine or cytokine receptors (Aruffo and Seed, (1987) supra).

Alternatively, CD28 might be a receptor which mediates cell-cell contact ("intercellular adhesion"). Antigen-independent intercellular interactions involving lymphocyte accessory molecules are essential for an immune response (Springer et al., (1987), supra). For example, binding of the T cell-associated protein, CD2, to its ligand LFA-3, a widely expressed glycoprotein (reviewed in Shaw and Shimuzu, Current Opinion in Immunology, Eds. Kindt and Long, 1:92-97 (1988)), is important for optimizing antigen-specific T cell activation (Moingeon et al., Nature 339:314 (1988)). Another important adhesion system involves binding of the

LFA-1 glycoprotein found on lymphocytes, macrophages, and granulocytes (Springer et al., (1987), supra; Shaw and Shimuzu (1988), supra) to its ligands ICAM-1 (Makgoba et al., Nature 331:86-88 (1988)) and ICAM-2 (Staunton et al., Nature 339:61-64 (1989)). The T cell accessory molecules CD8 and CD4 strengthen T cell adhesion by interaction with MHC class I (Norment et al., Nature 336:79-81 (1988)) and class II (Doyle and Strominger, Nature 330:256-259 (1987)) molecules, respectively. "Homing receptors" are important for control of lymphocyte migration (Stoolman, Cell 56:907-910 (1989)). The VLA glycoproteins are integrins which appear to mediate lymphocyte functions requiring adhesion to extracellular matrix components (Hemler, Immunology Today 9:109-113 (1988)). The CD2/LFA-3, LFA-1/ICAM-1 and ICAM-2, and VLA adhesion systems are distributed on a wide variety of cell types (Springer et al., (1987), supra; Shaw and Shimuzu, (1988,) supra and Hemler, (1988), supra).

Intercellular adhesion interactions mediated by integrins are strong interactions that may mask other intercellular adhesion interactions. For example, interactions mediated by integrins require divalent cations (Kishimoto et al., Adv. Immunol. 46:149-182 (1989)). These interactions may mask other intercellular adhesion interactions that are divalent cation independent. Therefore, it would be useful to develop assays that permit identification of non-integrin mediated ligand/receptor interactions.

T cell interactions with other cells such as B cells are essential to the immune response. Levels of many cohesive molecules found on T cells and B cells increase during an immune response (Springer et al., (1987), supra; Shaw and Shimuzu, (1988), supra; Hemler (1988), supra). Increased levels of these molecules may help explain why activated B

cells are more effective at stimulating antigen-specific T cell proliferation than are resting B cells (Kaiuchi et al., J. Immunol. 131:109-114 (1983); Kreiger et al., J. Immunol 135:2937-2945 (1985); McKenzie, J. Immunol. 141: 2907-2911 (1988); and Hawrylowicz and Unanue, J. Immunol. 141:4083-4088 (1988)). The fact that anti-CD28 mAbs inhibit mixed lymphocyte reactions (MLR) may suggest that the CD28 antigen is also an adhesion molecule.

Optimal activation of B lymphocytes and their subsequent differentiation into immunoglobulin secreting cells is dependent on the helper effects of major histocompatibility complex (MHC) class II antigen (Ag)-reactive CD4 positive T helper ($CD4^+$ T_h) cells and is mediated via both direct (cognate) T_h -B cell intercellular contact-mediated interactions and the elaboration of antigen-nonspecific cytokines (non-cognate activation; see, e.g. Noel and Snow, Immunol. Today 11:361 (1990)). Although T_h -derived cytokines can stimulate B cells (Moller, Immunol. Rev. 99:1 (1987)), their synthesis and directional exocytosis is initiated and sustained via cognate interactions between antigen-primed T_h cells and antigen-presenting B cells (Moller, supra). The successful outcome of T_h -B interactions requires participation of transmembrane receptor-ligand pairs of co-stimulatory accessory/adhesion molecules on the surface of T_h and B cells which include CD2 (LFA-2); CD58 (LFA-3), CD4:MHC class II, CD11a/CD18 (LFA-1):CD54 (ICAM-1).

During cognate T_h :B interaction, although both T_h and B cells cross-stimulate each other, their functional differentiation is critically dependent on the provision by T_h cells of growth and differentiation-inducing cytokines such as IL-2, IL-4 and IL-6 (Noel, supra, Kupfer et al., supra, Brian, supra and Moller, supra). Studies by Poo et al. (Nature 332:378 (1988)) on cloned T_h :B interaction indicate that

interaction of the T cell receptor complex (TCR) with nominal Ag-MHC class II on B cells results in focused release of T_h cell-derived cytokines in the area of T_h and B cell contact (vectorially oriented exocytosis). This may ensure the
5 activation of only B cells presenting antigen to T_h cells, and also avoids activation of bystander B cells.

It was proposed many years ago that B lymphocyte activation requires two signals (Bretscher and Cohn, Science
10 169:1042-1049 (1970)) and now it is believed that all lymphocytes require two signals for their optimal activation, an antigen specific or clonal signal, as well as a second, antigen non-specific signal (Janeway, supra). The signals
15 required for a T helper cell (T_h) antigenic response are provided by antigen-presenting cells (APC). The first signal is initiated by interaction of the T cell receptor complex (Weiss, J. Clin. Invest. 86:1015 (1990)) with antigen
20 presented in the context of class II major histocompatibility complex (MHC) molecules on the APC (Allen, Immunol. Today 8:270 (1987)). This antigen-specific signal is not sufficient to generate a full response, and in the absence of a second
25 signal may actually lead to clonal inactivation or anergy (Schwartz, Science 248:1349 (1990)). The requirement for a second "costimulatory" signal provided by the MHC has been demonstrated in a number of experimental systems (Schwartz, supra; Weaver and Unanue, Immunol. Today 11:49 (1990)). The
30 molecular nature of these second signal(s) is not completely understood, although it is clear in some cases that both soluble molecules such as interleukin (IL)-1 (Weaver and Unanue, supra) and membrane receptors involved in intercellular adhesion (Springer, Nature 346:425 (1990)) can provide costimulatory signals.

Freeman et al. (J. Immunol. 143(8):2714-2722 (1989)) isolated and sequenced a cDNA clone encoding a B cell activation antigen recognized by mAb B7 (Freeman et al., J. Immunol. 138:3260 (1987)). COS cells transfected with this cDNA have been shown to stain by both labeled mAb B7 and mAb BB-1 (Clark et al., Human Immunol. 16:100-113 (1986); Yokochi et al., J. Immunol. 128:823 (1981)); Freeman et al., (1989) supra; and Freedman et al., (1987), supra). Expression of the B cell activation antigen has been detected on cells of other lineages. For example, studies by Freeman et al. (1989) have shown that monocytes express low levels of mRNA for B7.

Expression of soluble derivatives of cell-surface glycoproteins in the immunoglobulin gene superfamily has been achieved for CD4, the receptor for HIV-1, using hybrid fusion molecules consisting of DNA sequences encoding portions of the extracellular domain of CD4 receptor fused to antibody domains (human immunoglobulin C gamma 1), as described by Capon et al., Nature 337:525-531 (1989).

While the CD28 antigen has functional and structural characteristics of a receptor, until now, a natural ligand for this molecule has not been identified. It would be useful to identify ligands that bind with the CD28 antigen and other receptors and to use such ligand(s) to regulate cellular responses, such as T cell and B cell interactions, for use in treating pathological conditions.

SUMMARY OF THE INVENTION

Accordingly, the present invention identifies the B7 antigen as a ligand recognized by the CD28 receptor. The B7 antigen, or its fragments or derivatives are reacted with CD28 positive T cells to regulate T cell interactions with other

cells. Alternatively, the CD28 receptor, its fragments or derivatives are reacted with B7 antigen to regulate interactions of B7 positive cells with T cells. In addition, antibodies or other molecules reactive with the B7 antigen or CD28 receptor may be used to inhibit interaction of cells associated with these molecules, thereby regulating T cell responses.

A preferred embodiment of the invention provides a method for regulating CD28 specific T cell interactions by reacting CD28 positive T cells with B7 antigen, or its fragments or derivatives, so as to block the functional interaction of T cells with other cells. The method for reacting a ligand for CD28 with T cells may additionally include the use of anti-CD monoclonal antibodies such as anti-CD2 and/or anti-CD3 monoclonal antibody.

In an alternative embodiment, the invention provides a method for regulating immune responses by contacting CD28 positive T cells with fragments containing at least a portion of the DNA sequence encoding the amino acid sequence corresponding to the extracellular domain of B7 antigen. In addition, derivatives of B7 antigen may be used to regulate immune responses, wherein the derivatives are fusion protein constructs including at least a portion of the extracellular domain of B7 antigen and another protein, such as human immunoglobulin C gamma 1, that alters the solubility, binding affinity and/or valency of B7 antigen. For example, in a preferred embodiment, DNA encoding amino acid residues from about position 1 to about position 215 of the sequence corresponding to the extracellular domain of B7 antigen is joined to DNA encoding amino acid residues of the sequences corresponding to the hinge, CH2 and CH3 regions of human Ig C γ 1 to form a DNA fusion product which encodes B7Ig fusion protein.

In another preferred embodiment, DNA encoding amino acid residues from about position 1 to about position 134 of the sequence corresponding to the extracellular domain of the CD28 receptor is joined to DNA encoding amino acid residues of the sequences corresponding to the hinge, CH2 and CH3 regions of human Ig C γ 1 to form a CD28Ig fusion protein.

Alternatively, fragments or derivatives of the CD28 receptor may be reacted with B cells to bind the B7 antigen and regulate T cell/B cell interactions. The methods for regulating T cell interactions may be further supplemented with the addition of a cytokine.

In another embodiment, the invention provides a method for treating immune system diseases mediated by T cell by administering B7 antigen, including B7Ig fusion protein, to react with T cells by binding the CD28 receptor.

In yet another embodiment, a method for inhibiting T cell proliferation in graft versus host disease is provided wherein CD28 positive T cells are reacted with B7 antigen, for example in the form of the B7Ig fusion protein, to bind to the CD28 receptor, and an immunosuppressant is administered.

The invention also provides a cell adhesion assay to identify ligands that interact with target receptors that mediate intercellular adhesion, particularly adhesion that is divalent cation independent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 are bar graphs showing the results of cellular adhesion experiments using CD28 positive (CD28⁺) and CD28 negative (CD28⁻) CHO cells as described in Example 1, infra.

Figure 2 are micrographs of the cellular adhesion studies of Figure 1, as described in Example 1, infra.

Figure 3 are bar graphs of experiments testing the ability of different human cell lines and normal and activated murine spleen B cells to adhere to CD28⁺ CHO cells, as described in Example 1, infra.

Figure 4 is a graph of the effects of blocking by mAbs on CD28-mediated adhesion to human B cells, as described in Example 1, infra.

Figure 5 is a bar graph of the results of adhesion between COS cells transfected with B7 antigen and CD28⁺ or CD28⁻ CHO cells, as described in Example 1, infra.

Figure 6 is a bar graph demonstrating the effect of anti-CD28 and anti-B7 mAbs on T cell proliferation as described in Example 2, infra.

Figure 7 is graphs showing the effects of DR7-primed CD4⁺CD45RO⁺ T_H cells on differentiation of B cells into immunoglobulin secreting cells, as described in Example 2, infra (7a: IgM production by SKW B cells; 7b: IgG production by CESS B cells).

Figure 8 is graphs showing the effect of anti-CD28 and anti-B7 mAbs on the T_H-induced production of immunoglobulin by B cells as described in Example 2, infra (8a: IgM production, 8b: IgG production).

Figure 9 is a diagrammatic representation of B7Ig (9a) and CD28Ig (9b) protein fusion constructs as described in Example 3, infra (dark shaded regions = oncostatin M; unshaded regions = B7 and CD28, stippled regions = human Ig Cγ1).

Figure 10 is a photograph of a gel obtained from purification of B7Ig and CD28 protein fusion constructs as described in Example 3, infra.

5 Figure 11 depicts the results of FACS^R analysis of binding of the B7Ig and CD28Ig fusion proteins to transfected CHO cells as described in Example 3, infra.

10 Figure 12 is a graph illustrating competition binding analysis of ¹²⁵I-labeled B7Ig fusion protein to immobilized CD28Ig fusion protein as described in Example 3, infra.

15 Figure 13 is a graph showing the results of Scatchard analysis of B7Ig fusion protein binding to immobilized CD28Ig fusion protein as described in Example 3, infra.

Figure 14 is a graph of FACS^R profiles of B7Ig fusion protein binding to PHA blasts as described in Example 3, infra.

20 Figure 15 is an autoradiogram of ¹²⁵I-labeled proteins immunoprecipitated by B7Ig as described in Example 3, infra.

25 Figure 16 is a graph showing the effect of B7Ig binding to CD28 on CD28-mediated adhesion as described in Example 3, infra.

30 Figure 17 is a photograph of the results of RNA blot analysis of the effects of B7 on accumulation of IL-2 mRNA as described in Example 3, infra.

DETAILED DESCRIPTION OF THE INVENTION

35 In order that the invention herein described may be more fully understood, the following description is set forth.

This invention is directed to the identification of a ligand reactive with CD28 antigen (hereafter referred to as "CD28 receptor"), and to methods of using the ligand and its fragments and derivatives, including fusion proteins. Also disclosed is a cell adhesion assay method to detect ligands for cell surface receptors.

Recently, Freeman et al., (J. Immunol. 143(8):2714-2722 (1989)) isolated and sequenced a cDNA clone encoding a B cell activation antigen recognized by monoclonal antibody (mAb) B7 (Freedman et al., J. Immunol. 139:3260 (1987)). COS cells transfected with this cDNA were shown to stain by both mAb B7 and mAb BB-1 (Clark et al., Human Immunology 16:100-113 (1986), and Yokochi et al., (1981), supra; Freeman et al., (1989) supra; and Freedman et al., (1987), supra). The ligand for CD28 was identified by the experiments described herein, as the B7/BB-1 antigen isolated by Freeman et al., (Freedman et al., and Freeman et al., supra, both of which are incorporated by reference herein).

For convenience, the ligand for CD28, identified as the B7/BB-1 antigen, is referred to herein as the "B7 antigen".

The term "fragment" as used herein means a portion of the amino acid sequence corresponding to the B7 antigen or CD28 receptor. For example, a fragment of the B7 antigen useful in the method of the present invention is a polypeptide containing a portion of the amino acid sequence corresponding to the extracellular portion of the B7 antigen, i.e. the DNA encoding amino acid residues from position 1 to 215 of the sequence corresponding to the B7 antigen described by Freeman et al., supra. A fragment of the CD28 antigen that may be used is a polypeptide containing amino acid residues from about position 1 to about position 134 of the sequence

corresponding to the CD28 receptor as described by Aruffo and Seed, Proc. Natl. Acad. Sci. (USA) 84:8573-8577 (1987).

5 The term "derivative" as used herein includes a fusion protein consisting of a polypeptide including portions of the amino acid sequence corresponding to the B7 antigen or CD28 antigen. For example, a derivative of the B7 antigen useful in the method of the present invention is a B7Ig fusion protein that comprises a polypeptide corresponding to the
10 extracellular domain of the B7 antigen and an immunoglobulin constant region that alters the solubility, affinity and/or valency (valency is defined herein as the number of binding sites available per molecule) of the B7 antigen.

15 The term "derivative" also includes monoclonal antibodies reactive with the B7 antigen or CD28 receptor, or fragments thereof, and antibodies reactive with the B7Ig and CD28Ig fusion proteins of the invention.

20 The B7 antigen and/or its fragments or derivatives for use in the present invention may be produced in recombinant form using known molecular biology techniques based on the cDNA sequence published by Freeman et al., supra. Specifically, cDNA sequences encoding the amino acid sequence
25 corresponding to the B7 antigen or fragments or derivatives thereof can be synthesized by the polymerase chain reaction (see U.S. Patent No. 4,683,202) using primers derived from the published sequence of the antigen (Freeman et al., supra). These cDNA sequences can then be assembled into a eukaryotic
30 or prokaryotic expression vector and the resulting vector can be used to direct the synthesis of the ligand for CD28 by appropriate host cells, for example COS or CHO cells. CD28 receptor and/or its fragments or derivatives may also be produced using recombinant methods.

In a preferred embodiment, DNA encoding the amino acid sequence corresponding to the extracellular domain of the B7 antigen, containing amino acids from about position 1 to about position 215, is joined to DNA encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of human Ig Cγ1, using PCR, to form a construct that is expressed as B7Ig fusion protein. DNA encoding the amino acid sequence corresponding to the B7Ig fusion protein has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland, under the Budapest Treaty on May 31, 1991 and accorded accession number 68627.

In another embodiment, DNA encoding the amino acid sequence corresponding to the extracellular domain of the CD28 receptor, containing amino acids from about position 1 to about position 134, is joined to DNA encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of human Ig Cγ1 using PCR to form a construct expressed as CD28Ig fusion protein. DNA encoding the amino acid sequence corresponding to the CD28Ig fusion protein has been deposited in the ATCC, in Rockville, Maryland under the Budapest Treaty on May 31, 1991 and accorded accession number 68628.

The techniques for assembling and expressing DNA encoding the amino acid sequences corresponding to B7 antigen and soluble B7Ig and CD28Ig fusion proteins, e.g synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like are well-established in the art, and most practitioners are familiar with the standard resource materials for specific conditions and procedures. However, the following paragraphs are provided for convenience and notation of modifications where necessary, and may serve as a guideline.

Cloning and Expression of Coding Sequences for Receptors and
Fusion Proteins

5 cDNA clones containing DNA encoding CD28 and B7
proteins are obtained to provide DNA for assembling CD28 and
B7 fusion proteins as described by Aruffo and Seed, Proc.
Natl. Acad. Sci. USA 84:8573-8579 (1987) (for CD28); and
Freeman et al., J. Immunol. 143:2714-2722 (1989) (for B7),
10 incorporated by reference herein. Alternatively, cDNA clones
may be prepared from RNA obtained from cells expressing B7
antigen and CD28 receptor based on knowledge of the published
sequences for these proteins (Aruffo and Seed, and Freeman,
supra) using standard procedures.

15 The cDNA is amplified using the polymerase chain
reaction ("PCR") technique (see U.S. Patent Nos. 4,683,195 and
4,683,202 to Mullis et al. and Mullis & Faloona, Methods
Enzymol. 154:335-350 (1987)) using synthetic oligonucleotides
20 encoding the sequences corresponding to the extracellular
domain of the CD28 and B7 proteins as primers. PCR is then
used to adapt the fragments for ligation to the DNA encoding
amino acid fragments corresponding to the human immunoglobulin
constant γ 1 region, i.e. sequences encoding the hinge, CH2
and CH3 regions of Ig C γ 1 to form B7Ig and CD28Ig fusion
25 constructs and to expression plasmid DNA to form cloning and
expression plasmids containing sequences corresponding to B7
or CD28 fusion proteins.

30 To produce large quantities of cloned DNA, vectors
containing DNA encoding the amino acid sequences corresponding
to the fusion constructs of the invention are transformed into
suitable host cells, such as the bacterial cell line MC1061/p3
using standard procedures, and colonies are screened for the
appropriate plasmids.

The clones obtained as described above are then transfected into suitable host cells for expression. Depending on the host cell used, transfection is performed using standard techniques appropriate to such cells. For example, transfection into mammalian cells is accomplished using DEAE-dextran mediated transfection, CaPO_4 co-precipitation, lipofection, electroporation, or protoplast fusion, and other methods known in the art including: lysozyme fusion or erythrocyte fusion, scraping, direct uptake, osmotic or sucrose shock, direct microinjection, indirect microinjection such as via erythrocyte-mediated techniques, and/or by subjecting host cells to electric currents. The above list of transfection techniques is not considered to be exhaustive, as other procedures for introducing genetic information into cells will no doubt be developed.

Expression plasmids containing cDNAs encoding sequences corresponding to CD28 and B7 for cloning and expression of CD28Ig and B7Ig fusion proteins include the OMCD28 and OMB7 vectors modified from vectors described by Aruffo and Seed, Proc. Natl. Acad. Sci. USA (1987), supra, (CD28); and Freeman et al., (1989), supra, (B7), both of which are incorporated by reference herein. Preferred host cells for expression of CD28Ig and B7Ig proteins include COS and CHO cells.

Expression in eukaryotic host cell cultures derived from multicellular organisms is preferred (see Tissue Cultures, Academic Press, Cruz and Patterson, Eds. (1973)). These systems have the additional advantage of the ability to splice out introns and thus can be used directly to express genomic fragments. Useful host cell lines include Chinese hamster ovary (CHO), monkey kidney (COS), VERO and HeLa cells. In the present invention, cell lines stably expressing the fusion constructs are preferred.

Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, CMV promoter (CDM8 vector) and avian sarcoma virus (ASV) (π LN vector). Other commonly used early and late promoters include those from Simian Virus 40 (SV 40) (Fiers, et al., Nature 273:113 (1973)), or other viral promoters such as those derived from polyoma, Adenovirus 2, and bovine papilloma virus. The controllable promoter, hMTIII (Karin, et al., Nature 299:797-802 (1982)) may also be used. General aspects of mammalian cell host system transformations have been described by Axel (U.S. Patent No. 4,399,216 issued Aug. 16, 1983). It now appears, that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in non-coding DNA regions. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eukaryotes.

Although preferred host cells for expression of the DNA constructs include eukaryotic cells such as COS or CHO cells, other eukaryotic microbes may be used as hosts. Laboratory strains of Saccharomyces cerevisiae, Baker's yeast, are most used although other strains such as Schizosaccharomyces pombe may be used. Vectors employing, for example, the 2 μ origin of replication of Broach, Meth. Enz. 101:307 (1983), or other yeast compatible origins of replications (see, for example, Stinchcomb et al., Nature 282:39 (1979)); Tschempe et al., Gene 10:157 (1980); and Clarke et al., Meth. Enz. 101:300 (1983)) may be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7:149 (1968); Holland et al., Biochemistry 17:4900 (1978)). Additional promoters known in the art include the CMV promoter provided in the CDM8 vector (Toyama and Okayama,

FEBS 268:217-221 (1990); the promoter for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255:2073 (1980)), and those for other glycolytic enzymes. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose utilization. It is also believed terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes.

Alternatively, prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of E. coli; however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta-lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., Nature 198: 1056 (1977)), the tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. 8:4057 (1980)) and the lambda derived P_L promoter and N-gene ribosome binding site (Shimatake et al., Nature 292:128 (1981)).

The nucleotide sequences encoding the amino acid sequences corresponding to the CD28Ig and B7Ig fusion proteins, may be expressed in a variety of systems as set forth below. The cDNA may be excised by suitable restriction enzymes and ligated into suitable prokaryotic or eukaryotic expression vectors for such expression. Because CD28 receptors occur in nature as dimers, it is believed that successful expression of these proteins requires an expression

system which permits these proteins to form as dimers. Truncated versions of these proteins (i.e. formed by introduction of a stop codon into the sequence at a position upstream of the transmembrane region of the protein) appear not to be expressed. The expression of CD28 antigen in the form of a fusion protein permits dimer formation of the protein. Thus, expression of CD28 antigen as a fusion product is preferred in the present invention.

Sequences of the resulting fusion protein constructs are confirmed by DNA sequencing using known procedures, for example as described by Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463 (1977) as further described by Messing et al., Nucleic Acids Res. 9:309 (1981) or by the method of Maxam et al. Methods Enzymol. 65:499 (1980)).

Recovery of Protein Products

As noted above, the CD28 receptor is not readily expressed as a mature protein using direct expression of DNA encoding the amino acid sequence corresponding to the truncated protein. To enable homodimer formation, it is preferred that DNA encoding the amino acid sequence corresponding to the extracellular domain of CD28 and including the codons for a signal sequence such as oncostatin M in cells capable of appropriate processing, is fused with DNA encoding amino acids corresponding to the Fc domain of a naturally dimeric protein. Purification of the fusion protein products after secretion from the cells is thus facilitated using antibodies reactive with the anti-immunoglobulin portion of the fusion proteins. When secreted into the medium, the fusion protein product is recovered using standard protein purification techniques, for example by application to protein A columns.

In addition to the fusion proteins of the invention, monoclonal antibodies reactive with the B7 antigen and CD28 receptor, and reactive with B7Ig and CD28Ig fusion proteins, may be produced by hybridomas prepared using known procedures, such as those introduced by Kohler and Milstein (see Kohler and Milstein, Nature, 256:495-97 (1975), and modifications thereof, to regulate cellular interactions.

These techniques involve the use of an animal which is primed to produce a particular antibody. The animal can be primed by injection of an immunogen (e.g. the B7Ig fusion protein) to elicit the desired immune response, i.e. production of antibodies reactive with the ligand for CD28, the B7 antigen, from the primed animal. A primed animal is also one which is expressing a disease. Lymphocytes derived from the lymph nodes, spleens or peripheral blood of primed, diseased animals can be used to search for a particular antibody. The lymphocyte chromosomes encoding desired immunoglobulins are immortalized by fusing the lymphocytes with myeloma cells, generally in the presence of a fusing agent such as polyethylene glycol (PEG). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques; for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653, Sp2/0-Ag14, or HL1-653 myeloma lines. These myeloma lines are available from the ATCC, Rockville, Maryland.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium such as HAT medium, in which unfused parental myeloma or lymphocyte cells eventually die. Only the hybridoma cells survive and can be grown under limiting dilution conditions to obtain isolated clones. The supernatants of the hybridomas are screened for the presence of the desired specificity, e.g. by immunoassay techniques using the B7Ig fusion protein that has been used

for immunization. Positive clones can then be subcloned under limiting dilution conditions, and the monoclonal antibody produced can be isolated.

5 Various conventional methods can be used for isolation and purification of the monoclonal antibodies so as to obtain them free from other proteins and contaminants. Commonly used methods for purifying monoclonal antibodies include ammonium sulfate precipitation, ion exchange
10 chromatography, and affinity chromatography (see Zola et al., in Monoclonal Hybridoma Antibodies: Techniques and Applications, Hurell (ed.) pp. 51-52 (CRC Press, 1982)). Hybridomas produced according to these methods can be propagated in vitro or in vivo (in ascites fluid) using
15 techniques known in the art (see generally Fink et al., Prog. Clin. Pathol., 9:121-33 (1984), Fig. 6-1 at p. 123).

 Generally, the individual cell line may be propagated in vitro, for example, in laboratory culture
20 vessels, and the culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration, or centrifugation.

 In addition, fragments of these antibodies
25 containing the active binding region of the extracellular domain of B7 or CD28 antigen, such as Fab, F(ab')₂ and Fv fragments, may be produced. Such fragments can be produced using techniques well established in the art (see e.g. Rousseaux et al., in Methods Enzymol., 121:663-69, Academic
30 Press (1986)).

USES

General

 The experiments described below in the Examples,
35 suggest that the CD28 receptor and its ligand, the B7 antigen,

may function in vivo by mediating T cell interactions with other cells such as B cells. The functional consequences of these interactions may be induced or inhibited using ligands that bind to the native CD28 receptor or the B7 antigen.

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It is expected that administration of the B7 antigen will result in effects similar to the use of anti-CD28 monoclonal antibodies (mAbs) reactive with the CD28 receptor in vivo. Thus, because anti-CD28 mAbs may exert either
10 stimulatory or inhibitory effects on T cells, depending, in part, on the degree of crosslinking or "aggregation" of the CD28 receptor (Damle, J. Immunol. 140:1753-1761 (1988); Ledbetter et al., Blood 75(7):1531-1539 (1990)) it is expected
15 that the B7 antigen, its fragments and derivatives, will act to stimulate or inhibit T cells in a manner similar to the effects observed for an anti-CD28 monoclonal antibody, under similar conditions in vivo. For example, administration of B7 antigen, e.g. as a soluble B7Ig fusion protein to react with CD28 positive T cells, will bind the CD28 receptor on the
20 T cells and result in inhibition of the functional responses of T cells. Under conditions where T cell interactions are occurring as a result of contact between T cells and B cells, binding of introduced B7 antigen in the form of a fusion protein that binds to CD28 receptor on CD28 positive T cells
25 should interfere, i.e. inhibit, the T cell interactions with B cells. Likewise, administration of the CD28 antigen, or its fragments and derivatives in vivo, for example in the form of a soluble CD28Ig fusion protein, will result in binding of the soluble CD28Ig to B7 antigen, preventing the endogenous
30 stimulation of CD28 receptor by B7 positive cells such as activated B cells, and interfering with the interaction of B7 positive cells with T cells.

Alternatively, based on the known effects of aggregating the CD28 receptor, either by reacting T cells with immobilized ligand, or by crosslinking as described by Ledbetter et al., Blood 75(7):1531-1539 (1990)), the B7 antigen, and/or its fragments or derivatives, may be used to stimulate T cells, for example by immobilizing B7 antigen or B7Ig fusion protein, for reacting with the T cells. The activated T cells stimulated in this manner in vitro may be used in vivo in adoptive therapy.

Therefore, the B7 antigen and/or fragments or derivatives of the antigen may be used to react with T cells to regulate immune responses mediated by functional T cell responses to stimulation of the CD28 receptor. The B7 antigen may be presented for reaction with CD28 positive T cells in various forms. Thus, in addition to employing activated B cells expressing the B7 antigen, the B7 antigen may be encapsulated, for example in liposomes, or using cells that have been genetically engineered, for example using gene transfer, to express the antigen for stimulation of the CD28 receptor on T cells.

The CD28 receptor, and/or its fragments or derivatives, may also be used to react with cells expressing the B7 antigen, such as B cells. This reaction will result in inhibition of T cell activation, and inhibition of T cell dependent B cell responses, for example as a result of inhibition of T cell cytokine production.

In an additional embodiment of the invention, other reagents, such as molecules reactive with B7 antigen or the CD28 receptor are used to regulate T and/or B cell responses. For example, antibodies reactive with the CD28Ig fusion proteins, and Fab fragments of CD28Ig, may be prepared using the CD28Ig fusion protein as immunogen, as described above.

These anti-CD28 antibodies may be screened to identify those capable of inhibiting the binding of the B7 antigen to CD28 antigen. The antibodies or antibody fragments such as Fab fragments may then be used to react with the T cells, for example, to inhibit CD28 positive T cell proliferation.

The use of Fab fragments of the 9.3 monoclonal antibody, or Fab fragments of the anti-CD28Ig monoclonal antibodies as described herein, is expected to prevent binding of CD28 receptor on T cells to B7 antigen, for example on B cells. This will result in inhibition of the functional response of the T cells.

Similarly, anti-B7 monoclonal antibodies such as BB-1 mAb, or anti-B7Ig monoclonal antibodies prepared as described above using B7Ig fusion protein as immunogen, may be used to react with B7 antigen positive cells such as B cells to inhibit B cell interaction via the B7 antigen with CD28 positive T cells.

In another embodiment the B7 antigen may be used to identify additional compounds capable of regulating the interaction between the B7 antigen and the CD28 antigen. Such compounds may include soluble fragments of the B7 antigen or CD28 antigen or small naturally occurring molecules that can be used to react with B cells and/or T cells. For example, soluble fragments of the ligand for CD28 containing the extracellular domain (e.g. amino acids 1-215) of the B7 antigen may be tested for their effects on T cell proliferation.

Uses In Vitro and In Vivo

In a method of the invention, the ligand for CD28, B7 antigen, is used for regulation of CD28 positive (CD28⁺) T cells. For example, the B7 antigen is reacted with T cells in

vitro to crosslink or aggregate the CD28 receptor, for example using CHO cells expressing B7 antigen, or immobilizing B7 on a solid substrate, to produce activated T cells for administration in vivo for use in adoptive therapy. In
5 adoptive therapy T lymphocytes are taken from a patient and activated in vitro with an agent. The activated cells are then reinfused into the autologous donor to kill tumor cells (see Rosenberg et al., Science 223:1318-1321 (1986)). The method can also be used to produce cytotoxic T cells useful in
10 adoptive therapy as described in copending U.S. Patent application serial no. 471,934, filed January 25, 1990, incorporated by reference herein.

Alternatively, the ligand for CD28, its fragments or
15 derivatives, may be introduced in a suitable pharmaceutical carrier in vivo, i.e. administered into a human subject for treatment of pathological conditions such as immune system diseases or cancer. Introduction of the ligand in vivo is
20 expected to result in interference with T cell/B cell interactions as a result of binding of the ligand to T cells. The prevention of normal T cell/B cell contact may result in decreased T cell activity, for example, decreased T cell proliferation.

In addition, administration of the B7 antigen in vivo is expected to result in regulation of in vivo levels of cytokines, including, but not limited to, interleukins, e.g. interleukin ("IL")-2, IL-3, IL-4, IL-6, IL-8, growth factors including tumor growth factor ("TGF"), colony stimulating
30 factor ("CSF"), interferons ("IFNs"), and tumor necrosis factor ("TNF") to promote desired effects in a subject. It is anticipated that ligands for CD28 such as B7Ig fusion proteins and Fab fragments may thus be used in place of cytokines such as IL-2 for the treatment of cancers in vivo. For example,
35 when the ligand for CD28 is introduced in vivo it is available

to react with CD28 antigen positive T cells to mimic B cell contact resulting in increased production of cytokines which in turn will interact with B cells.

5 Under some circumstances, as noted above, the effect of administration of the B7 antigen, its fragments or derivatives in vivo is stimulatory as a result of aggregation of the CD28 receptor. The T cells are stimulated resulting in an increase in the level of T cell cytokines, mimicking the effects of T cell/B cell contact on triggering of the CD28 antigen on T cells. In other circumstances, inhibitory effects may result from blocking by the B7 antigen of the CD28 triggering resulting from T cell/B cell contact. For example, the B7 antigen may block T cell proliferation. Introduction of the B7 antigen in vivo will thus produce effects on both T and B cell mediated immune responses. The ligand may also be administered to a subject in combination with the introduction of cytokines or other therapeutic reagents. Alternatively, for cancers associated with the expression of B7 antigen, such as B7 lymphomas, carcinomas, and T cell leukemias, ligands reactive with the B7 antigen, such as anti-B7Ig monoclonal antibodies, may be used to inhibit the function of malignant B cells.

25 Because CD28 is involved in regulation of the production of several cytokines, including TNF and gamma interferon (Lindsten et al., supra, (1989)), the ligand for CD28 of the invention may be useful for in vivo regulation of cytokine levels in response to the presence of infectious agents. For example, the ligand for CD28 may be used to increase antibacterial and antiviral resistance by stimulating tumor necrosis factor (TNF) and IFN production. TNF production seems to play a role in antibacterial resistance at early stages of infection (Havell, J. Immunol. 143:2894-2899 (1990)). In addition, because herpes virus infected cells are

more susceptible to TNF-mediated lysis than uninfected cells (Koff and Fann, Lymphokine Res. 5:215 (1986)), TNF may play a role in antiviral immunity.

5 Gamma interferon is also regulated by CD28 (Lindsten et al., supra). Because mRNAs for alpha and beta IFNs share potential regulatory sequences in their 3' untranslated regions with cytokines regulated by CD28, levels of these cytokines may also be regulated by the ligand for CD28. Thus,
10 the ligand for CD28 may be useful to treat viral diseases responsive to interferons (De Maeyer and De Maeyer-Guignard, in Interferons and Other Regulatory Cytokines, Wiley Publishers, New York (1988)). Following the same reasoning, the ligand for CD28 may also be used to substitute for alpha-
15 IFN for the treatment of cancers, such as hairy cell leukemia, melanoma and renal cell carcinoma (Goldstein and Laszio, CA: a Cancer Journal for Clinicians 38:258-277 (1988)), genital warts and Kaposi's sarcoma.

20 In addition, B7Ig fusion proteins as described above may be used to regulate T cell proliferation. For example, the soluble CD28Ig and B7Ig fusion proteins may be used to block T cell proliferation in graft versus host (GVH) disease which accompanies allogeneic bone marrow transplantation. The CD28-
25 mediated T cell proliferation pathway is cyclosporine-resistant, in contrast to proliferation driven by the CD3/Ti cell receptor complex (June et al., 1987, supra). Cyclosporine is relatively ineffective as a treatment for GVH disease (Storb, Blood 68:119-125 (1986)). GVH disease is
30 thought to be mediated by T lymphocytes which express CD28 antigen (Storb and Thomas, Immunol. Rev. 88:215-238 (1985)). Thus, the B7 antigen in the form of B7Ig fusion protein, or in combination with immunosuppressants such as cyclosporine, for blocking T cell proliferation in GVH disease. In addition,
35 B7Ig fusion protein may be used to crosslink the CD28

receptor, for example by contacting T cells with immobilized B7Ig fusion protein, to assist in recovery of immune function after bone marrow transplantation by stimulating T cell proliferation.

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The fusion proteins of the invention may be useful to regulate granulocyte macrophage colony stimulating factor (GM-CSF) levels for treatment of cancers (Brandt et al., N. Eng. J. Med. 318:869-876 (1988)), AIDS (Groopman et al., N. Eng. J. Med. 317:593-626 (1987)) and myelodysplasia (Vadan-Raj et al., N. Eng. J. Med. 317:1545-1551 (1987)).

15 Regulation of T cell interactions by the methods of the invention may thus be used to treat pathological conditions such as autoimmunity, transplantation, infectious diseases and neoplasia.

20 In a preferred embodiment, the role of CD28-mediated adhesion in T cell and B cell function was investigated using procedures used to demonstrate intercellular adhesion mediated by MHC class I (Norment et al., (1988) supra) and class II (Doyle and Strominger, (1987) supra) molecules with the CD8 and CD4 accessory molecules, respectively. The CD28 antigen was expressed to high levels in Chinese hamster ovary (CHO) cells and the transfected cells were used to develop a CD28-mediated cell adhesion assay, described infra. With this assay, an interaction between the CD28 antigen and its ligand expressed on activated B lymphocytes, the B7 antigen, was demonstrated. The CD28 antigen, expressed in CHO cells, was shown to mediate specific intracellular adhesion with human lymphoblastoid and leukemic B cell lines, and with activated murine B cells. CD28-mediated adhesion was not dependent upon divalent cations. A mAb, BB-1, reactive with B7 antigen was shown to inhibit CD28-mediated adhesion. Transfected COS cells expressing the B7 antigen were also shown to adhere to

CD28⁺ CHO cells; this adhesion was blocked by mAbs to CD28 receptor and B7 antigen. The specific recognition by CD28 receptor of B7 antigen, indicated that B7 antigen is the ligand for the CD28 antigen.

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The results presented herein also demonstrate that antibodies reactive with CD28 and B7 antigen specifically block helper T_H-mediated immunoglobulin production by allogeneic B cells, providing evidence of the role of CD28/B7 interactions in the collaboration between T and B cells.

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In additional preferred embodiments, B7Ig and CD28Ig fusion proteins were constructed by fusing DNA encoding the extracellular domains of B7 antigen or the CD28 receptor to DNA encoding portions of human immunoglobulin C gamma 1. These fusion proteins were used to further demonstrate the interaction of the CD28 receptor and its ligand, the B7 antigen.

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The cell adhesion assay method of the invention permits identification and isolation of ligands for target cell surface receptors mediating intercellular adhesion, particularly divalent cation independent adhesion. The target receptor may be an antigen or other receptor on lymphocytes such as T or B cells, on monocytes, on microorganisms such as viruses, or on parasites. The method is applicable for detection of ligand involved in ligand/receptor interactions where the affinity of the receptor for the ligand is low, such that interaction between soluble forms of the ligand and target receptor is difficult to detect. In such systems, adhesion interactions between other ligands and receptors that are divalent cation dependent may "mask" other interactions between ligands for target receptors, such that these interactions are only observed when divalent cations are removed from the system.

The cell adhesion assay utilizes cells expressing target cell surface receptor and cells to be tested for the presence of ligand mediating adhesion with the receptor. The cells expressing target receptor may be cells that are transfected with the receptor of interest, such as Chinese hamster ovary (CHO) or COS cells. The cells to be tested for the presence of ligand are labeled, for example with ^{51}Cr , using standard methods and are incubated in suitable medium containing a divalent cation chelating reagent such as ethylenediamine tetraacetic acid (EDTA) or ethyleneglycol tetraacetic acid (EGTA). Alternatively, the assay may be performed in medium that is free of divalent cations, or is rendered free of divalent cations, using methods known in the art, for example using ion chromatography. Use of a divalent cation chelating reagent or cation-free medium removes cation-dependent adhesion interactions permitting detection of divalent cation-independent adhesion interactions. The labeled test cells are then contacted with the cells expressing target receptor and the number of labeled cells bound to the cells expressing receptor is determined by measuring the label, for example using a gamma counter. A suitable control for specificity of adhesion can be used, such as a blocking antibody, which competes with the ligand for binding to the target receptor.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the disclosure or the protection granted by Letters Patent hereon.

EXAMPLE 1

Identification of the Ligand For CD28 Receptor

5 If CD28 receptor antigen binds to a cell surface
ligand, then cells expressing the ligand should adhere more
readily to cells expressing CD28 receptor than to cells which
do not. To test this, a cDNA clone encoding CD28 under
control of a highly active promoter (Aruffo and Seed, (1987)
10 supra) together with a selectable marker (pSV2dhfr) (Mulligan
and Berg, Science 209:1414-1422 (1980)) was transfected into
dihydrofolate reductase (dhfr)-deficient CHO cells.

15 Cell Culture. T51, 1A2, 5E1, Daudi, Raji, Jijoye, CEM,
Jurkat, HSB2, THP-1 and HL60 cells (Bristol-Myers Squibb
Pharmaceutical Research Institute, Seattle, WA) were cultured
in complete RPMI medium (RPMI containing 10% fetal bovine
serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.
Dhfr-deficient Chinese hamster ovary (CHO) cells (Urlaub and
20 Chasin, Proc. Natl. Acad. Sci., 77:4216-4220 (1980)) were
cultured in Maintenance Medium (Ham's F12 medium (GIBCO, Grand
Island, NY) supplemented with 10% FBS, 0.15 mM L-proline, 100
U/ml penicillin and 100 µg/ml streptomycin). Dhfr-positive
transfectants were selected and cultured in Selective Medium
25 (DMEM, supplemented with 10% FBS, 0.15 mM L-proline, 100 U/ml
penicillin and 100 µg/ml streptomycin).

Spleen B cells were purified from Balb/c mice by
treatment of total spleen cells with an anti-Thy 1.2 mAb
30 (30H12) (Ledbetter and Herzenberg, Immunol. Rev. 47:361-389
(1979)) and baby rabbit complement. The resulting
preparations contained approximately 85% B cells, as judged by
FACS^R analysis following staining with fluorescein
isothiocyanate (FITC)-conjugated goat anti-mouse
35 immunoglobulin (TAGO). These cells were activated by

treatment for 72 hrs with E. coli lipopolysaccharide (LPS, List Biological Laboratories, Campbell, CA) at 10 µg/ml in complete RPMI.

5 Monoclonal Antibodies. Monoclonal antibody (mAb) 9.3 (anti-CD28) (ATCC No. HB 10271, Hansen et al., Immunogenetics 10:247-260 (1980)) was purified from ascites before use. mAb 9.3 F(ab')₂ fragments were prepared as described by Parham, in J. Immunol. 131:2895-2902 (1983). Briefly, purified mAb 9.3
10 was digested with pepsin at pH 4.1 for 75 min. followed by passage over protein A Sepharose to remove undigested mAb. A number of mAbs to B cell-associated antigens were screened for their abilities to inhibit CD28-mediated adhesion. mAbs 60.3 (CD18); 1F5 (CD20); G29-5 (CD21); G28-7, HD39, and HD6 (CD22);
15 HD50 (CD23); KB61 (CD32); G28-1 (CD37); G28-10 (CD39); G28-5 (CD40); HERMES1 (CD44); 9.4 (CD45); LB-2 (CD54) and 72F3 (CD71) have been previously described and characterized in International Conferences on Human Leukocyte Differentiation Antigens I-III (Bernard et al., Eds., Leukocyte Typing,
20 Springer-Verlag, New York (1984); Reinherz et al., Eds., Leukocyte Typing II Vol. 2 New York (1986); and McMichael et al., Eds., Leukocyte Typing III Oxford Univ. Press, New York, (1987)). These mAbs were purified before use by protein A Sepharose chromatography or by salt precipitation and in
25 exchange chromatography. δTA401 (Kuritani and Cooper, J. Exp. Med. 155:839-848 (1982)) (Anti-IgD); 2C3 (Clark et al., (1986), supra) (anti-IgM); Namb1, H1DE, P10.1, W6/32 (Clark et al., (1986) supra; and Gilliland et al., Human Immunology 25:269-289 (1989), anti-human class I); and HB10A (Clark et
30 al., (1986), supra, anti-MHC class II) were also purified before use. mAbs B43 (CD19); BL-40 (CD72); AD2, 1E9.28.1, and 7G2.2.11 (CD73); EBU-141, LN1 (CDw75); CRIS-1 (CD-76); 424/4A11, 424/3D9 (CD77) Leu 21, Ba, 1588, LO-panB-1, FN1, and FN4 (CDw78); and M9, G28-10, HuLym10, 2-7, F2B2.6, 121, L26,
35 HD77, NU-B1, BLAST-1, BB-1, anti-BL7, anti-HC2, and L23 were

used as coded samples provided to participants in the Fourth International Conference on Human Leukocyte Differentiation Antigens (Knapp, Ed., Leukocyte Typing IV, Oxford Univ. Press, New York (1990)). These were used in ascites form. mAbs BB-1 and LB-1 (Yokochi et al., (1981), supra) were also purified from ascites before use. Anti-integrin receptor mAbs P3E3, P4C2, P4G9 (Wayner et al., J. Cell. Biol. 109:1321-1330 (1989)) were used as hybridoma culture supernatants.

Immunostaining Techniques. For indirect immunofluorescence, cells were incubated with mAbs at 10 µg/ml in complete RPMI for 1 hr at 4°C. mAb binding was detected with a FITC-conjugated goat anti-mouse immunoglobulin second step reagent. For direct binding experiments, mAbs 9.3 and BB-1 were directly conjugated with FITC as described by Goding in Monoclonal Antibodies: Principles and Practices Academic Press, Orlando, FL (1983), and were added at saturating concentrations in complete RPMI for 1 hr at 4°C. Non-specific binding of FITC-conjugated mAbs was measured by adding the FITC conjugate following antigen pre-blocking (20-30 min at 4°C) with unlabeled mAb 9.3 or BB-1. Immunohistological detection of adherent lymphoblastoid cells was achieved using the horseradish peroxidase (HRP) method described by Hellstrom et al., J. Immunol. 127:157-160 (1981).

Plasmids and Transfections. cDNA clones encoding the amino acid sequences corresponding to T cell antigens CD4, CD5 and CD28 in the expression vector p π H3M (Aruffo and Seed (1987), supra), were provided by Drs. S. Aruffo and B. Seed, Massachusetts General Hospital, Boston, MA. An expressible cDNA clone in the vector CDM8 encoding the amino acid sequence corresponding to B7 antigen (Freeman et al., J. Immunol. 143:2714-2722 (1989)) was provided by Dr. Gordon Freeman (Dana Farber Cancer Institute, Boston, MA).

Dhfr-deficient CHO cells were co-transfected with a mixture of 9 μ g of plasmid π H3M-CD28 (Aruffo and Seed, (1987) supra) and 3 μ g of plasmid pSV2dhfr (Mulligan and Berg, (1980), supra) using the calcium phosphate technique (Graham and Van Der Eb, Virology 52:456-467 (1973)). Dhfr-positive colonies were isolated and grown in Selective Medium containing increasing amounts of methotrexate (Sigma Chemical Co., St. Louis, MO). Cells resistant to 10 nM methotrexate were collected by incubation in PBS containing 10 mM EDTA, stained for presence of the CD28 receptor by indirect immunofluorescence, and separated by FACS^R into CD28-positive (CD28⁺) and CD28-negative (CD28⁻) populations. Both populations were again cultured in Selective Medium containing increasing concentrations of methotrexate to 1 μ M, stained for the CD28 antigen and again sorted into CD28⁺ and CD28⁻ populations.

COS cells were transfected with B7, CD4 or CD5 cDNAs as described by Malik et al., Molecular and Cellular Biology 9:2847-2853 (1989). Forty-eight to seventy-two hours after transfection, cells were collected by incubation in PBS containing 10 mM EDTA, and used for flow cytometry analysis or in CD28-mediated adhesion assays as described, infra.

Cell lines expressing high (CD28⁺) and low (CD28⁻) levels of the CD28 receptor were isolated from amplified populations by FACS^R sorting following indirect immunostaining with mAb 9.3. After two rounds of FACS^R selection, the CD28⁺ population stained uniformly positive with FITC-conjugated mAb 9.3 (mean channel, 116 in linear fluorescence units), while the CD28⁻ population stained no brighter (mean channel, 3.9) than unstained cells (mean channel, 3.7). Staining by CD28⁺ CHO cells was approximately ten-fold brighter than phytohemagglutinin-stimulated T cells (mean channel, 11.3). The CD28⁺ and CD28⁻ populations stably maintained their phenotypes

after more than 6 months of continuous culture in Selective Medium containing 1 μ M of methotrexate.

Cell Adhesion Assay for a Ligand For CD28

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An adhesion assay to detect differential binding to CD28⁺ and CD28⁻ CHO cells by cells expressing a ligand for CD28 was developed. Since mAb 9.3 has been shown to inhibit mixed lymphocyte reactions using B lymphoblastoid cells lines as a source of alloantigen (Damle et al., (1981) supra; and Lesslauer et al., Eur. J. Immunol. 16:1289-1296 (1986)) B lymphoblastoid cell lines were initially tested for CD28-mediated adhesion.

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CD28-Mediated Adhesion Assay. Cells to be tested for adhesion were labeled with ⁵¹Cr (0.2-1 mCi) to specific activities of 0.2-2 cpm/cell. A mouse mAb having irrelevant specificity, mAb W1, directed against human breast carcinoma-associated mucin, (Linsley et al., Cancer Res. 46:5444-5450 (1986)), was added to the labeling reaction to a final concentration of 100 μ g/ml to saturate Fc receptors. Labeled and washed cells were preincubated in complete RPMI containing 10 μ g/ml of mAb W1, and unless otherwise indicated, 10 mM EDTA. mAb 9.3 or mAb 9.3 F(ab')₂ was added to some samples at 10 μ g/ml, for approximately 1 hr at 23°C.

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Labeled cells (1-10 x 10⁶/well in a volume of 0.2 ml complete RPMI, containing EDTA and mAbs, where indicated) were then added to the CHO monolayers. Adhesion was initiated by centrifugation in a plate carrier (1,000 rpm, in a Sorvall HB1000 rotor, approximately 210 X g) for 3 min at 4°C. Plates were then incubated at 37°C for 1 hr. Reactions were terminated by aspirating unbound cells and washing five times with cold, complete RPMI. Monolayers were solubilized by addition of 0.5 N NaOH, and radioactivity was measured in a

gamma counter. For most experiments, numbers of bound cells were calculated by dividing total bound radioactivity (cpm) by the specific activity (cpm/cell) of labeled cells. When COS cells were used, their viability at the end of the experiment was generally less than 50%, so specific activity calculations were less accurate. Therefore, for COS cells results are expressed as cpm bound.

In pilot experiments, T51 lymphoblastoid cells were found to adhere more to CD28⁺ CHO cells, than to CD28⁻ CHO cells. Furthermore, adhesion of T51 cells to CD28⁺ CHO cells was partially blocked by mAb 9.3, while adhesion to CD28⁻ CHO cells was not consistently affected. Adhesion was not affected by control mAb L6 (ATCC No. HB 8677, Hellstrom et al., Cancer Res. 46:3917-3923 (1986)), which is of the same isotype as mAb 9.3 (IgG2a). These experiments suggested that T51 cells adhered specifically to CD28⁺ CHO cells. Since blocking of adhesion by mAb 9.3 was incomplete, ways to increase the specificity of the CD28 adhesion assay were explored.

The effects of divalent cation depletion on T51 cell adhesion to CD28⁺ and CD28⁻ CHO cells were examined. Preliminary experiments showed that EDTA treatment caused loss of CHO cells during washing, so the CHO cell monolayers were fixed with paraformaldehyde prior to EDTA treatment. Fixation did not significantly affect CD28-mediated adhesion by T51 cells either in the presence or absence of mAb 9.3. Monolayers of CD28⁺ and CD28⁻ CHO cells (1 to $1.2 \times 10^5/\text{cm}^2$ in 48 well plastic dishes) were fixed in 0.5% paraformaldehyde for 20 min at 23°C, washed and blocked in Complete RPMI for 1 hr, then pre-incubated with or without mAb 9.3 or mAb 9.3 F(ab')₂ at 10 µg/ml in Complete RPMI for 1 hr at 37°C. T51 cells were labeled with ⁵¹Cr, preincubated with or without 10 mM EDTA, added to CHO cells and cellular adhesion was

measured. The results are presented in Figure 1. Mean and standard deviation (error bars) are shown for three replicate determinations.

5 The specificity of CD28-mediated adhesion was greatly increased in the presence of EDTA (Figure 1). Adhesion to CD28⁺ cells in the presence of EDTA was 17-fold greater than to CD28⁻ cells in the presence of EDTA, compared with 5.5-fold greater in its absence. Adhesion to CD28⁺ cells
10 in the presence of mAb 9.3 plus EDTA was reduced by 93%, compared with 62% in the presence of mAb alone. CD28-mediated adhesion of T51 cells in the presence of EDTA could also be seen quite clearly by microscopic examination following immunohistological staining of T51 cells. Cellular adhesion
15 between unlabeled T51 cells and CD28⁺ or CD28⁻ CHO cells was determined in the presence of 10 mM EDTA as described above. Adherent T51 cells were stained with biotinylated anti-human Class II Ab, HB10a, fixed with 0.2% glutaraldehyde and visualized by sequential incubation with avidin-conjugated HRP
20 (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine solution (Hellstrom and Hellstrom, J. Immunol. 127:157-160 (1981)). The results of staining are shown in Figure 2. A similar, but slightly less significant increase in adhesion specificity, was also observed in the
25 presence of the calcium-specific chelator, EGTA.

The Ligand For CD28 is a B Cell Activation Marker

30 The increased specificity of CD28-mediated adhesion in EDTA made it possible to more readily detect adhesion by cells other than T51. A number of additional cell lines were tested, including three lymphoblastoid lines (T51, 1A2, and 5E1); four Burkett's Lymphoma lines (Daudi, Raji, Jijoye, and Namalwa); one acute lymphoblastic (B cell) leukemia (REH);
35 three T cell leukemias (CEM, Jurkat and HSB2); and two

monocytic leukemias (THP-1 and HL60). As a source of primary B cells, murine splenic B cells, before and after activation with LPS, were tested. All cells were tested for adhesion to both CD28⁺ and CD28⁻ CHO cells, in the absence and presence of MAb 9.3. The cells were labeled with ⁵¹Cr and CD28-mediated adhesion was measured as described above. Three representative experiments showing adhesion to CD28⁺ CHO cells are shown in Figure 3. Inhibition by mAb 9.3 is shown as an indicator of specificity; in most cases, adhesion measured in the presence of mAb 9.3 was approximately equal to adhesion to CD28⁺ cells.

CD28-specific adhesion (i.e., adhesion being greater than 70% inhibitable by mAb 9.3), was observed with T51, 5E1, Raji, and Jijoye cells. Daudi cells also showed specific adhesion, although to a lesser extent. Other cell lines did not show specific CD28-mediated adhesion, although some (e.g., Namalwa) showed relatively high non-specific adhesion. Primary mouse splenic B cells did not show CD28-mediated adhesion, but acquired the ability to adhere following activation with LPS. In other experiments, six additional lymphoblastoid lines showed CD28-mediated adhesion, while the U937 cell line, unstimulated human tonsil B cells, and phytohemagglutinin stimulated T cells did not show adhesion. These experiments indicate that a ligand for CD28 is found on the cell surface of activated B cells of human or mouse origin.

CD28-Mediated Adhesion is Specifically Blocked by a mAb (BB-1) to B7 Antigen

In initial attempts to define B cell molecules involved in CD28-mediated adhesion, adhesion by lymphoblastoid cell lines having mutations in other known cellular adhesion molecules was measured using the adhesion assay described

above. The 616 lymphoblastoid line (MHC class II-deficient) (Gladstone and Pious, Nature 271:459-461 (1978)) bound to CD28⁺ CHO cells equally well or better than parental T51 cells. Likewise, a CD18-deficient cell line derived from a patient with leukocyte adhesion deficiency (Gambaro cells) (Beatty et al., Lancet 1:535-537 (1984)) also adhered specifically to CD28. Thus, MHC class II and CD18 molecules do not mediate adhesion to CD28.

A panel of mAbs to B cell surface antigens were then tested for their ability to inhibit CD28-mediated adhesion of T51 cells. For these experiments, a total of 57 mAbs reactive with T51 cells were tested, including mAbs to the B cell-associated antigens CD19, CD20, CD21, CD22, CD23, CD37, CD39, CD40, CD71, CD72, CD73, CDw75, CD76, CD77, CDw78, IgM, and IgD; other non-lineage-restricted antigens CD18, CD32, CD45, CD54, and CD71; CD44 and another integrin; MHC class I and class II antigens; and 30 unclustered B cell associated antigens. In addition to these, many other mAbs which did not react with T51 by FACS^R analysis were tested. Initial screening experiments were carried out in the absence of EDTA, and any mAbs which blocked adhesion were subsequently retested in the presence and absence of EDTA. Of these mAbs, only those directed against MHC class I molecules (Namb1, H1DE, P10.1, W6/32), and one to an unclustered B cell antigen (BB-1), originally described as a B cell activation marker (Yokochi et al., (1981) supra) were consistently able to block CD28-mediated adhesion by greater than 30%.

The dose-dependence of adhesion inhibition by the anti-Class I mAb, H1DE, by BB-1 and by 9.3 were compared in the presence of EDTA in the experiment shown in Figure 4. Jijoye cells were labeled with ⁵¹Cr and allowed to adhere to CD28⁺ CHO cells in the presence of 10 mM EDTA as described above. Adhesion measured in the presence of the indicated

amounts of mAbs 9.3, H1DE (anti-human class I MHC, Gaur et al., Immunogenetics 27:356-361 (1988)), or mAb BB-1 is expressed as a percentage of maximal adhesion measured in the absence of mAb (45,000 cells bound). mAb 9.3 was most effective at blocking, but mAb BB-1 was able to block approximately 60% of adhesion at concentrations less than 1 μ g/ml. mAb H1DE also partially blocked adhesion at all concentrations tested. When EDTA was omitted from the adhesion assay, blocking by class I mAbs was consistently less, and required higher mAb concentrations, than mAbs 9.3 or BB-1.

Binding of mAb BB-1 by Different Cells Correlates With CD28-Specific Adhesion

To investigate the roles of molecules recognized by anti-class I and BB-1 mAbs in CD28-mediated adhesion, levels of these antigens on certain of the cell lines tested for CD28-specific adhesion in Figure 3 were compared. Cells were analyzed by FACS^R following indirect immunofluorescence staining with mAbs H1DE and BB-1. Cell lines 1A2, Namalwa, REH and HL60 (which did not adhere specifically to CD28) all bound high levels of mAb H1DE, whereas Daudi cells (which did adhere) did not show detectable binding. Therefore, a direct correlation between CD28-mediated adhesion and expression of class I antigens was not observed. On the other hand, these experiments suggested a correlation between adhesion to CD28 and staining by mAb BB-1.

To confirm this correlation, cell lines examined for CD28-mediated adhesion in Figure 3 were tested for staining by direct immunofluorescence using FITC-conjugated mAb BB-1 (Table 1). Cell lines were incubated with no mAb or with FITC-conjugated mAb BB-1 with or without preincubation with cold (unlabeled) BB-1 mAb. Values shown in Table 1 represent

mean fluorescence in linear units. All of the cell lines which adhered specifically to CD28 receptor (Figure 3) bound higher levels of the FITC-conjugate than those which did not adhere specifically. Antigen specificity was demonstrated in all cases by the ability of unlabeled mAb BB-1 to compete for binding of the FITC-conjugate.

Table 1

CELLS WHICH ADHERE TO CD28 RECEPTOR ALSO BIND mAb BB-1

			<u>FITC-BB-1</u>		<u>SPECIFIC⁴</u>
<u>LINE</u>	<u>CELL TYPE¹</u>	<u>NO mAb</u>	<u>-COLD</u>	<u>+COLD</u>	<u>BINDING</u>
Positive for CD28 Adhesion ²					
T51	B-LCL	2.3	16.4	3.0	13.4
5E1	B-LCL	2.1	13.0	2.4	10.6
Jijoye	BL	2.3	17.8	2.8	15.0
Raji	BL	2.1	7.1	2.8	4.3
Daudi	BL	2.1	6.4	2.8	3.6
Negative for CD28 Adhesion ³					
1A2	B-LCL	2.1	4.5	4.3	<1
Namalwa	BL	2.2	3.8	2.2	1.6
REH	B-ALL	2.0	2.1	2.0	<1
CEM	T-ALL	2.3	2.0	1.9	<1
Jurkat	T-ALL	2.2	2.2	2.0	<1
HSB2	T-ALL	2.1	2.3	2.3	<1
HL60	AML	2.3	3.1	3.1	<1
THP-1	AML	2.3	3.1	3.0	<1

¹ B-LCL = B-lymphoblastoid cell line

BL = Burkett's lymphoma

B-ALL = B cell-derived acute lymphoblastoid leukemia

T-ALL = T cell-derived acute lymphoblastoid leukemia

AML = Acute Monocytic leukemia

² Positive for CD28 adhesion = >70% inhibition of adhesion by mAb 9.3

³ Negative for CD28 Adhesion = < 70% inhibition of adhesion by mAb 9.3.

⁴ Specific binding = (FITC-BB-1 + cold) subtracted from (FITC BB-1 - cold).

COS Cells Expressing the B7 Antigen Adhere Specifically to
CD28

The role of the B7 antigen recognized by mAb BB-1 in
5 CD28-mediated adhesion was investigated using a cDNA clone
isolated and sequenced by Freeman et al. as described in J.
Immunol. 143:2714-2722 (1989). COS cells were transfected
with an expression vector containing the cDNA clone encoding
10 the B7 antigen, as described by Freeman et al., (1989), supra
as described above. Forty-eight hours later, transfected COS
cells were removed from their dishes by incubation in PBS
containing 10 mM EDTA, and were labeled with ⁵¹Cr. Cells were
shown to express B7 antigen by FACS^R analysis following
indirect staining with mAb BB-1 as reported by Freeman et al,
15 supra. Adhesion between B7 transfected COS cells and CD28⁺ or
CD28⁻ CHO cells was then measured in the presence of 10 mM EDTA
as described above. Where indicated, adhesion was measured in
the presence of mAbs 9.3 or BB-1 (10 µg/ml). As shown in
Figure 5, B7/BB-1-transfected COS cells adhered readily to
20 CD28⁺ CHO cells; adhesion was completely blocked by both mAbs
9.3 and BB-1. No adhesion to CD28⁻ CHO cells was detected.
This experiment was repeated five times with identical
results.

25 In other experiments, adhesion was not blocked by
non-reactive, isotype matched controls, mAb W5 (IgM) (Linsley,
(1986) supra) and mAb L6 (IgG2A) (Hellstrom et al., (1986)
supra), or by mAb H1DE, which reacts with class I antigens on
COS cells. CD28-mediated adhesion by B7 transfected cells
30 could also be clearly seen by microscopic examination of the
CHO cell monolayers after the assay. When COS cells were
transfected with expressible CD4 or CD5 cDNA clones, no CD28-
mediated adhesion was detected. Expression of CD4 and CD5 was
confirmed by FACS^R analysis following immunofluorescent
35 staining. When EDTA was omitted from the assay, adhesion

measured with CD5-transfected COS cells was greatly increased but not inhibited by mAb 9.3. In contrast, adhesion by B7 transfected COS cells under these conditions was still partially blocked (approximately 40%) by mAb 9.3. Thus, transfection of B7 into COS cells confers the ability on the cells to adhere specifically to CD28 receptor.

The above assay for intracellular adhesion mediated by the CD28 receptor, described above, demonstrated CD28-mediated adhesion by several lymphoblastoid and leukemic B cell lines, and by primary murine spleen cells following activation with LPS. These results indicate the presence of a natural ligand for the CD28 receptor on the cell surface of some activated B lymphocytes.

Several lines of evidence show that the B cell molecule which interacted with the CD28 receptor is the B7 antigen. mAb BB-1 was identified from a panel of mAbs as the mAb which most significantly inhibited CD28-mediated adhesion. Furthermore, a correlation was observed between the presence of B7 antigen and CD28-mediated adhesion (Table 1). Finally, COS cells transfected with B7 cDNA demonstrated CD28-mediated adhesion. Taken together, these observations provide strong evidence that B7 antigen is a ligand for CD28 receptor. Because both CD28 (Aruffo and Seed, (1987) supra) and B7 (Freeman et al., (1989) supra) are members of the immunoglobulin superfamily, their interaction represents another example of heterophilic recognition between members of this gene family (Williams and Barclay (1988), supra).

CD28-mediated adhesion differs in several respects from other cell adhesion systems as shown in the above results. CD28-mediated adhesion was not blocked by mAbs to other adhesion molecules, including mAbs to ICAM-1 (LB-2), MHC class II (HB10a) CD18 (60.3), CD44 (HERMES-1 homing receptor),

and an integrin (P3E3, P4C2, P4G9). CD28-mediated adhesion was also resistant to EDTA and EGTA, indicating that this system does not require divalent cations, in contrast to integrins (Kishimoto et al., Adv. Immunol. 46:149-182 (1989)) and some homing receptors (Stoolman, Cell 56:907-910 (1989)) which require divalent cations. In the system described herein, in which CD28 receptor was expressed to high levels relative to those on activated T cells, it was sometimes difficult to measure CD28-mediated adhesion because of cation-dependent "background" adhesion (i.e., that not blocked by MAb 9.3, see Figure 1). Preliminary experiments suggest that background adhesion in the absence of EDTA was also blocked by MAb 60.3, which inhibits adhesion mediated by LFA-1 (Pohlman et al., J. Immunol. 136:4548-4553 (1986)). Even under optimal conditions, some cells (such as Namalwa, see Figure 3) showed significant non-CD28 dependent adhesion to CHO cells. Non-CD28 mediated adhesion systems may also be responsible for the incomplete blockage by mAb BB-1 of B cell adhesion (Figure 4). That this mAb is more effective at blocking adhesion by transfected COS cells (Figure 5) may indicate that non-CD28 mediated systems are less effective in COS cells.

Finally, CD28-mediated adhesion appears more restricted in its cellular distribution to T and B cells as compared to other adhesion molecules. CD28 receptor is primarily expressed by cells of the T lymphocyte lineage. The B7 antigen is primarily expressed by cells of the B lymphocyte lineage. Consistent with this distribution, the ligand for CD28 was only detected on cells of B lymphocyte lineage. Thus, available data suggest that CD28 mediates adhesion mainly between T cells and B cells. However, since CD28 expression has been detected on plasma cells (Kozbor et al., J. Immunol 138:4128-4132 (1987)) and B7 on cells of other lineages, such as monocytes (Freeman et al., (1989) supra), it is possible that other cell types may also employ this system.

Many adhesion molecules are known to mediate T cell-B cell interactions during an immune response and the levels of several of these, including CD28 and B7 antigen, have been reported to increase following activation. Increased levels of these molecules may help explain why activated B cells are more effective at stimulating antigen-specific T cell proliferation than are resting B cells. Because the B7 antigen is not expressed on resting B cells, CD28-mediated adhesion may play a role in maintaining or amplifying the immune response, rather than initiating it. Such a role is also consistent with the function of CD28 in regulating lymphokine and cytokine levels (Thompson et al., (1989), supra; and Lindsten et al., (1989), supra).

EXAMPLE 2

Characterization of Interaction Between CD28 Receptor and B7 Antigen

This example used alloantigen-driven maturation of B cells as a model system to demonstrate the involvement of the CD28 receptor on the surface of major histocompatibility complex (MHC) class II antigen-reactive CD4 positive T helper (T_h) cells and antigen presenting B cells during the T_h -B cell cognate interaction leading to B cell differentiation into immunoglobulin-secreting cells (IgSC).

Cognate interaction between $CD4^+$ T_h and antigen-presenting B cells results in the activation and differentiation of both cell types consequently leading to the development of immunoglobulin-secreting cells (Moller (Ed) Immunol Rev. 99:1 (1987), supra). Allogenic MLR offers an ideal system to analyze cognate T_h -B cell interaction because alloantigen-specific $CD4^+$ T_h induce both the activation and differentiation of alloantigen-bearing B cells into immunoglobulin secreting cells (Chiorazzi et al., Immunol Rev.

45:219 (1979); Kotzin et al., J. Immunol. 127:931 (1981);
Friedman et al., J. Immunol. 129:2541 (1982); Goldberg et al.,
J. Immunol. 135:1012 (1985); and Crow et al., J. Exp. Med.
164:1760 (1986)). The involvement of the CD28 receptor on T_h
5 cells and its ligand B7 during the activation of T_h and B cells
in the allogeneic MLR was first examined using murine mAb
directed at these molecules.

Culture medium. Complete culture medium (CM) consisted
10 of RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented
with 100 U/ml of penicillin G, 100 µg/ml of streptomycin, 2 mM
L-glutamine, 5 X 10⁻⁵ M 2-ME, and 10% FBS (Irvine Scientific).

Cells and mAbs. EBV-transformed B cell lines CESS (HLA-
15 AS1, A3; B5, B17; DR7), JIJOYE, and SKW6.4 (HLA-A1a; B27, B51;
DR7), were obtained from the ATCC. EBV-transformed B cell
lines ARENT (HLA-A2; B38, B39, DRw6) and MSAB (HLA-A1, A2;
B57; DR7) were provided by Dr. E. G. Engleman, Stanford
University School of Medicine, Stanford, CA. Hybridomas OKT4
20 (IgG anti-CD4), OKT8 (IgG anti-CD8) and HNK1 (IgM anti-CD57)
were obtained from the ATCC and ascitic fluids from these
hybridomas were generated in pristane-primed BALB/c mice.
Production and characterization of anti-CD28 mAb 9.3 (IgG2a)
has been described by Ledbetter et al., J. Immunol. 135:2331
25 (1985); Hara et al., J. Exp. Med. 161:1513 (1985) and Martin
et al., J. Immunol. 136:3282 (1986), incorporated by reference
herein. mAb 4H9 (IgG2a anti-CD7) as described by Damle and
Doyle, J. Immunol. 143:1761 (1989), incorporated by reference
herein, was provided by Dr. Engleman and mAb anti-B7 antibody
30 (BB1; IgM) as described by Tokochi et al., J. Immunol. 128:823
(1981), incorporated by reference herein, was provided by Dr.
E. Clark, University of Washington, Seattle, WA.

Peripheral blood mononuclear cells (PBMC) from healthy donors were separated into T and non-T cells using a sheep erythrocyte rosetting technique, and T cells were separated by panning into CD4⁺ subset and further into CD4⁺CD45RA - CD45RO⁺ memory subpopulation as described by Damle et al., J. Immunol. 139:1501 (1987), incorporated by reference herein.

Proliferative responses of T cells. To examine the effect of anti-CD28 and anti-B7 mAbs on the proliferative responses of T cells, fifty-thousand CD4⁺CD45RO⁺ T cells were stimulated by culturing with 1 x 10⁴ irradiated (8000 rad from a ¹³⁷Cs source) EBV-transformed allogenic B cells (or 2.5 X 10⁴ non-T cells) in 0.2 ml of CM in round-bottom microtiter wells in a humidified 5% CO₂ and 95% air atmosphere in the presence of 10 µg/ml of mAb reactive with CD7, CD28, CD57 or B7 antigen. CD4⁺CD45RO⁺ T cells also were also independently stimulated with 100 µg/ml of soluble purified protein derivative of tuberculin (PPD, Connough Laboratories, Willowdale, Ontario, Canada) in the presence of 1 X 10⁴ irradiated (3000 rad) autologous non-T cells in the presence of the above mAbs. Triplicate cultures were pulsed with 1 µCi/well = 37 kBq/well of [³H]dThd (6.7 Ci/mmol, NEN, Boston, MA) for 16 h before harvesting of cells for measurement of radiolabel incorporation into newly synthesized DNA. The results are expressed as cpm ± SEM. Proliferative responses were examined on day 7 of culture. EBV-transformed B cell lines were used as stimulator cells in these experiments because these B cells exhibit various features of activated B cells such as the expression of high levels of MHC class II and B7 molecules (Freeman et al., J. Immunol. 139:3260 (1987); and Yokochi et al., J. Immunol. 128:823 (1981)).

Figure 6 shows the results of these experiments. The presence of anti-CD28 mAb (9.3 IgG2a) but not that of isotype-matched anti-CD7 mAb (4H9, IgG2a) consistently inhibited the MLR proliferative response of CD4⁺ T cells to allogeneic B cells. Similarly, the addition of anti-B7 mAb (BB1; IgM) but not that of isotype-matched anti-CD57 HNK1; IgM) to the allogeneic MLR resulted in the inhibition of T cell proliferation. The inhibitory effects of anti-CD28 mAb 9.3 on the MLR responses of T cells are consistent with previous observations reported by Damle et al., J. Immunol. 120:1753 (1988) and Damle et al., Proc. Natl. Acad. Sci. USA 78:5096 (1981). Similar to the allogeneic MLR, proliferative response of CD4⁺ T cells to soluble Ag PPD presented by autologous non-T cells was also inhibited by anti-CD28 and anti-B7 mAb. Although both anti-CD28 mAb 9.3 (IgG2a) and anti-B7 mAb, BB1 (IgM) inhibited the allogeneic MLR and the soluble antigen-induced proliferative responses, anti-CD28-mediated inhibition was always stronger than that by anti-B7 for all the responder-stimulator combinations examined. These observations are also consistent with the weaker ability of anti-B7 mAb to block the CD28-mediated adhesion to B7⁺ B cells as described above.

T cell-induced Immunoglobulin (Ig) production by B cells

To further examine the roles of CD28 and B7 during cognate T_h-B interactions, two EBV-transformed B cells lines, IgG-secreting DR7⁺ CESS and IgM-secreting DR7⁺ SKW were used. When appropriately stimulated, both these B cells lines significantly increase their production of the respective Ig isotype. First, the effects of DR7-specific CD4⁺ CD45RO⁺ T_h line on the Ig production of both CESS and SKW B cells was examined. DR7-primed CD4⁺ T_h cells were derived from the allogeneic MLC consisting of responder CD4⁺CD45RO⁺ T cells (HLA-A26, A29; B7, B55; DR9, DR10) and irradiated MSAB (DR7⁺) B

cells as stimulator cells as described by Damle et al., J. Immunol, 133:1235 (1984), incorporated by reference herein. The isolation of resting CD4⁺CD45RO⁺ T cells and that of DR7-primed CD4⁺ CD45RO⁺ T lymphoblasts using discontinuous Percoll density gradient centrifugation was also as described by Damle, supra (1984). These DR7-primed CD4⁺ T_h cells were continuously propagated in the presence of irradiated MSAB B cells and 50 U/ml of IL-2. Prior to their functional analysis, viable DR7-primed T_h cells were isolated by Ficoll-Hypaque gradient centrifugation and maintained overnight in CM without DR7⁺ feeder cells or IL-2, after which immunoglobulin secreted in the cell-free supernatant (SN) was quantitated using a solid-phase ELISA.

To examine the effect of T_h cells on Ig production, by both CESS and SKW B cells 2 x 10⁴-2.5 x 10⁴ cells from HLA-DR7⁺ EBV-transformed B cell lines, IgM-producing SKW or IgG-producing CESS were cultured with varying numbers of DR7-primed CD4⁺CD45RO⁺ T_h cells for 96 h after which cell-free SN from these cultures were collected and assayed for the quantitation of IgM (SKW cultures) or IgG (CESS cultures) using solid-phase ELISA. Exogenous IL-6 (1-100 U/ml) induced Ig production by these B cells was also used as a positive control to monitor the non-cognate Ig production by these B cell lines. Ig production by freshly isolated resting CD4⁺CD45RO⁺ T_h cells (autologous to the DR7-primed CD4⁺ T_h cells) was also simultaneously examined as a control for DR7-primed CD4⁺ T_h cells.

Ig quantitation. IgG or IgM in culture SN were measured using solid-phase ELISA as described by Volkman et al., Proc. Natl. Acad. Sci. USA 78:2528 (1981), incorporated by reference herein. Briefly, 96-well flat-bottom microtiter ELISA plates (Corning, Corning NY) were coated with 200 µl/well of sodium carbonate buffer (pH 9.6) containing 10 µg/ml of affinity-

purified goat anti-human Ig or IgM Ab (Tago, Burlingame, CA) incubated overnight at 4° C, and then washed with PBS and wells were further blocked with 2% BSA in PBS (BSA-PBS). Samples to be assayed were added at appropriate dilution to these wells and incubated with 200 μ l/well of 1:1000 dilution of horseradish peroxidase (HRP)-conjugated F(ab')₂ fraction of affinity-purified goat anti-human IgG or IgM Ab (Tago). The plates were then washed, and 100 μ l/well of o-phenylenediamine (Sigma, St. Louis, MO) solution (0.6 mg/ml in citrate-phosphate buffer with pH 5.5 and 0.045% hydrogen peroxide). Color development was stopped with 2N sulfuric acid. Absorbance at 490 nm was measured with an automated ELISA plate reader. Test and control samples were run in triplicate and the values of absorbance were compared to those obtained with known IgG or IgM standards run simultaneously with the SN samples to generate the standard curve using which the concentrations of Ig in culture SN were quantitated. Data are expressed as ng/ml of Ig \pm SEM of either triplicate or quadruplicate cultures.

Figure 7 shows the Ig production by either B cell line as a function of the concentration of DR7-primed T_h with optimal Ig production induced at either 1:1 or 1:2 T_h:B ratios. At T_h:B ratios higher than 1:1 inhibition of Ig production was observed. Hence, all further experiments were carried out using a T_h:B ratio of 1:2. As shown in Figure 7, these unprimed resting CD4⁺ T_h cells slightly induced IgM production by SKW B cells but has no effect on the IgG production by CESS B cells in 4-day cultures. This slight helper effect observed with unprimed CD4⁺CD45RO⁺ population during the Ig induction cultures. The production of Ig by CESS (IgG) or SKW (IgM) B cells induced by DR7-primed CD4⁺ T_h was specific for HLA-DR7 because similarly activated DRw6-primed CD4⁺ T_h (stimulated with DRw6⁺ ARENT B cells and autologous to the DR7-primed T_h)

were unable to induce Ig production by either CESS or SKW B cells.

The roles of CD28 and B7 during cognate T_h :B-induced Ig production were further examined using anti-CD28 and anti-B7 mAbs. Both CESS and SKW B cells constitutively express B7 antigen on their surface and thus, represent a source of uniformly activated B cell populations for use in T_h -B cognate interactions or in cytokine-driven non-cognate maturation.

Thus, DR7⁺ B cells (CESS or SKW) were cultured for 4 days with DR7-specific CD4⁺ T_h line at T_h :B ratio of 1:2 and mAb to CD28 and B7, (and CD7 and CD57 as controls) were added to these cultures at different concentrations. Ig production (IgM, Figure 8a and IgG, Figure 8b) at the end of 3-day cultures was quantitated in cell-free SN. Figure 8 shows that both anti-CD28 and anti-B7 mAbs but not their isotype-matched mAb controls (anti-CD7 and anti-CD57, respectively) inhibited T_h induced Ig production by B cells in a dose-dependent manner. Once again, anti-CD28 mAb-mediated inhibition of Ig production was stronger than that by anti-B7 mAb. In contrast, Ig production by either B cells induced by exogenous IL-6 (non-cognate differentiation) was not affected by any of the above mAb.

These results strongly suggest that the interaction between CD28 and B7, during cognate T_h -B collaboration, in addition to activation of T_h cells, is pivotal to the differentiation of activated B cells into Ig secreting cells.

The above results demonstrate the relationship of CD28 receptor and its ligand, the B7 antigen, as a co-stimulatory transmembrane receptor-ligand pair influencing T_h :B interactions. Involvement of both CD28 and B7 during T_h :B collaboration was demonstrated by inhibition by anti-CD28 and anti-B7 of not only T_h cell activation but also T_h -induced

differentiation of B cells into IgSC. It appears as if the observed inhibitory effects of anti-CD28 and anti-B7 mAbs are due to the inhibition of CD28:B7 interaction underlying these responses.

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Interaction between CD28 receptor and B7 antigen may influence the production of cytokines and thus B cell differentiation. Ligation of CD28 by B7 during T_h :B collaboration may facilitate sustained synthesis and delivery of cytokines for their utilization during the differentiation of B cells into immunoglobulin secreting cells. The lack of inhibition by anti-CD28 and anti-B7 mAbs of cell dependent differentiation of CESS or SKW B cells induced with exogenous IL-4 or IL-6 suggests that CD28:B7 interaction controls either production of these cytokines, or their targeted delivery to B cells, or both of these events.

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The interaction of CD28 and B7 is most likely not restricted to T_h :B cell interactions, and applies more generally to other antigen-presenting cells such as monocyte/M ϕ , dendritic cells, and epidermal Langerhans cells. Ligation of a nominal antigen presented in conjunction with MHC class II molecules on the surface of antigen-presenting cells by the TcR/CD3 complex on the surface of T_h cells may lead to elevated expression of B7 antigen by these cells, which, via the interaction with CD28, then facilitates the production of various cytokines by T_h . This in turn drives both growth and differentiation of both T_h and B cells.

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EXAMPLE 3

Characterization of the Interaction between CD28 Receptor
and B7 Antigen

I. Preparation of Fusion Proteins

To further characterize the biochemical and functional aspects of the interactions between the CD28 receptor and B7 antigen, fusion proteins of B7 and CD28 with human immunoglobulin C gamma 1 (human Ig Cγ1) chains were constructed and expressed and used to measure the specificity and apparent affinity of interaction between these molecules. Purified B7Ig fusion protein, and CHO cells transfected with B7 antigen were used to investigate the functional effects of this interaction on T cell activation and cytokine production.

Preparation of B7Ig and CD28Ig Fusion Proteins

B7Ig and CD28Ig fusion proteins were prepared as follows. DNA encoding the amino acid sequence corresponding to the extracellular domain of the respective protein (B7 and CD28) was joined to DNA encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin Cγ1. This was accomplished as follows.

Plasmid Construction. Expression plasmids were used containing cDNA encoding the amino acid sequence corresponding to CD28 (pCD28) as described by Aruffo and Seed, Proc. Natl. Acad. Sci. USA 84:8573 (1987), incorporated by reference, and provided by Drs. Aruffo and Seed, Mass General Hospital, Boston, MA. Expression plasmids containing cDNA encoding the amino acid sequence corresponding to CD5 (pCD5) as described by Aruffo, Cell 61:1303 (1990), and also provided by Dr. Aruffo, and cDNA encoding the amino acid sequence corresponding to B7 (pB7) as described by Freeman et al., J.

Immunol. 143:2714 (1989)) and provided by Dr. Freeman, Dana Farber Cancer Institute, Boston, MA, were also used.

For initial attempts at expression of soluble forms of CD28 and B7, constructs were made (OMCD28 and OMB7) in which stop codons were introduced upstream of the transmembrane domains and the native signal peptides were replaced with the signal peptide from oncostatin M (Malik et al., Mol. Cell Biol. 9:2847 (1989)). These were made using synthetic oligonucleotides for reconstruction (OMCD28) or as primers (OMB7) for PCR. OMCD28, is a CD28 cDNA modified for more efficient expression by replacing the signal peptide with the analogous region from oncostatin M. CD28Ig and B7Ig fusion constructs were made in two parts. The 5' portions were made using OMCD28 and OMB7 as templates and the oligonucleotide, CTAGCCACTGAAGCTTCACCATGGGTGTACTGCTCACAC (SEQ ID NO:1) (corresponding to the oncostatin M signal peptide) as a forward primer, and either TGGCATGGGCTCCTGATCAGGCTTAGAAGGTCCGGGAAA (SEQ ID NO:2), or, TTTGGGCTCCTGATCAGGAAAATGCTCTTGCTTGGTTGT (SEQ ID NO:3) as reverse primers, respectively. Products of the PCR reactions were cleaved with restriction endonucleases (Hind III and BclI) as sites introduced in the PCR primers and gel purified.

The 3' portion of the fusion constructs corresponding to human Ig C γ 1 sequences was made by a coupled reverse transcriptase (from Avian myeloblastosis virus; Life Sciences Associates, Bayport, NY)-PCR reaction using RNA from a myeloma cell line producing human-mouse chimeric mAb L6 (provided by Dr. P. Fell and M. Gayle, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) as template. The oligonucleotide, AAGCAAGAGCATTTTCCTGATCA GGAGCCCAAATCTTCTGACAAACTCACACATCCCCACCGTCCCCAGCACCTGAACTCCTG (SEQ ID NO:4), was used as forward primer, and CTTGACCAAGTCTAGAAGCATCCTCGTGCGACCGCGAGAGC (SEQ ID NO:5) as

reverse primer. Reaction products were cleaved with BclI and XbaI and gel purified. Final constructs were assembled by ligating HindIII/BclI cleaved fragments containing CD28 or B7 sequences together with BclI/XbaI cleaved fragment containing Ig C γ 1 sequences into HindIII/XbaI cleaved CDM8. Ligation products were transformed into MC1061/p3 *E. coli* cells and colonies were screened for the appropriate plasmids. Sequences of the resulting constructs were confirmed by DNA sequencing. The DNA used in the B7 construct encodes amino acids from about position 1 to about position 215 of the sequence corresponding to the extracellular domain of the B7 antigen, and for CD28, the DNA encoding amino acids from about position 1 to about position 134 of the sequence corresponding to the extracellular domain of the CD28 receptor.

CD5Ig was constructed in identical fashion, using CATTGCACAGTCAAGCTTCCATGCCCATGGGTCTCTGGCCACCTTG (SEQ ID NO:6), as forward primer and ATCCACAGTGACAGTGATCATTGGATCCTGGCATGTGAC (SEQ ID NO:7) as reverse primer. The PCR product was restriction endonuclease digested and ligated with the Ig C γ 1 fragment as described above. The resulting construct (CD5Ig) encodes an amino acid sequence containing residues from about position 1 to about position 347 of CD5, two amino acids introduced by the construction procedure (amino acids DQ), followed by the Ig C γ 1 hinge region.

In initial attempts to make soluble derivatives of B7 and CD28, cDNA constructs were made encoding molecules truncated at the NH₂-terminal side of their transmembrane domains. In both cases, the native signal peptides were replaced with the signal peptide from oncostatin M (Malik, *supra*, 1989), which mediates efficient release of secreted proteins in transient expression assays. The cDNAs were cloned into an expression vector, transfected into COS cells, and spent culture medium was tested for secreted forms of B7

and CD28. In this fashion, several soluble forms of B7 were produced, but in repeated attempts, soluble CD28 molecules were not detected.

5 The next step was to construct receptor Ig Cyl fusion proteins. The DNAs encoding amino acid sequences corresponding to B7 and CD28 extracellular regions, preceded by the signal peptide to oncostatin M, were fused in frame to an Ig Cyl cDNA, as shown in Figure 9. During construction,
10 the Ig hinge disulfides were mutated to serine residues to abolish intrachain disulfide bonding. The resulting fusion proteins were produced in COS cells and purified by affinity chromatography on immobilized protein A as described below. Yields of purified protein were typically 1.5-4.5 mg/liter of
15 spent culture medium.

Polymerase Chain Reaction (PCR). For PCR, DNA fragments were amplified using primer pairs as described below for each fusion protein. PCR reactions (0.1 ml final volume) were run
20 in Tag polymerase buffer (Stratagene, La Jolla, CA), containing 20 μ moles each of dNTP; 50-100 pmoles of the indicated primers; template (1 ng plasmid or cDNA synthesized from $\leq 1 \mu$ g total RNA using random hexamer primer, as described by Kawasaki in PCR Protocols, Academic Press, pp.
25 21-27 (1990), incorporated by reference herein); and Tag polymerase (Stratagene). Reactions were run on a thermocycler (Perkin Elmer Corp., Norwalk, CT) for 16-30 cycles (a typical cycle consisted of steps of 1 min at 94°C, 1-2 min at 50°C and 1-3 min at 72°C).

30 Cell Culture and Transfections. COS (monkey kidney cells) were transfected with expression plasmids using a modification of the protocol of Seed and Aruffo (Proc. Natl. Acad. Sci. 84:3365 (1987)), incorporated by reference herein.
35 Cells were seeded at 10^6 per 10 cm diameter culture dish 18-24

h before transfection. Plasmid DNA was added (approximately 15 μ g/dish) in a volume of 5 ml of serum-free DMEM containing 0.1 mM cloroquine and 600 μ g/ml DEAE Dextran, and cells were incubated for 3-3.5 h at 37°C. Transfected cells were then briefly treated (approximately 2 min) with 10% dimethyl sulfoxide in PBS and incubated at 37°C for 16-24 h in DMEM containing 10% FCS. At 24 h after transfection, culture medium was removed and replaced with serum-free DMEM (6 ml/dish). Incubation was continued for 3 days at 37°C, at which time the spent medium was collected and fresh serum-free medium was added. After an additional 3 days at 37°C, the spent medium was again collected and cells were discarded.

CHO cells expressing CD28, CD5 or B7 were isolated as described by Linsley et al., (1991) supra, as follows: Briefly, stable transfectants expressing CD28, CD5, or B7, were isolated following cotransfection of dihydrofolate reductase-deficient Chinese hamster ovary (dhfr⁻ CHO) cells with a mixture of the appropriate expression plasmid and the selectable marker, pSV2dhfr, as described above in Example 1. Transfectants were then grown in increasing concentrations of methotrexate to a final level of 1 μ M and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1 μ M methotrexate. CHO lines expressing high levels of CD28 (CD28⁺ CHO) or B7 (B7⁺ CHO) were isolated by multiple rounds of fluorescence-activated cell sorting (FACS^R) following indirect immunostaining with mAbs 9.3 or BB-1. Amplified CHO cells negative for surface expression of CD28 or B7 (dhfr⁺ CHO) were also isolated by FACS^R from CD28-transfected populations.

Immunostaining and FACS^R Analysis. Transfected CHO cells or activated T cells were analyzed by indirect immunostaining. Before staining, CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells

were first incubated with murine mAbs 9.3 (Hansen et al.,
Immunogenetics 10:247 (1980)) or BB-1 (Yokochi et al., supra)
at 10 μ g/ml, or with Ig fusion proteins (CD28Ig, B7Ig, CD5Ig
or chimeric mAb L6 containing Ig C γ 1, all at 10 μ g/ml in DMEM
5 containing 10% FCS) for 1-2 h at 4°C. Cells were then washed,
and incubated for an additional 0.5-2h at 4°C with a FITC-
conjugated second step reagent (goat anti-mouse Ig serum for
murine mAbs, or goat anti-human Ig C γ serum for fusion
proteins (Tago, Inc., Burlingame, CA). Fluorescence was
10 analyzed on 10,000 stained cells using a FACS IV^R cell sorter
(Becton Dickinson and Co., Mountain View, CA) equipped with a
four decade logarithmic amplifier.

Purification of Ig Fusion Proteins. The first, second
15 and third collections of spent serum-free culture media from
transfected COS cells were used as sources for the
purification of Ig fusion proteins. After removal of cellular
debris by low speed centrifugation, medium was applied to a
column (approximately 200-400 ml medium/ml packed bed volume)
20 of immobilized protein A (Repligen Corp., Cambridge, MA)
equilibrated with 0.05 M sodium citrate, pH 8.0. After
application of the medium, the column was washed with 1 M
potassium phosphate, pH 8, and bound protein was eluted with
0.05 M sodium citrate, pH 3. Fractions were collected and
25 immediately neutralized by addition of 1/10 volume of 2 M
Tris, pH 8. Fractions containing the peak of A₂₈₀ absorbing
material were pooled and dialyzed against PBS before use.
Extinction coefficients of 2.4 and 2.8 ml/mg for CD28Ig and
B7Ig, respectively, by amino acid analysis of solutions of
30 known absorbance. The recovery of purified CD28Ig and B7Ig
binding activities were nearly quantitative as judged by FACS^R
analysis after indirect fluorescent staining of B7⁺ and CD28⁺
CHO cells.

SDS Page. SDS-PAGE was performed on linear acrylamide gradients gels with stacking gels of acrylamide. Aliquots (1 μ g) of B7Ig (lanes 1 and 3 of Figure 10) or CD28Ig (lanes 2 and 4) were subjected to SDS-PAGE (4-12% acrylamide gradient) under nonreducing (-BME, lanes 1 and 2) or reducing (+BME, lanes 3 and 4) conditions. Lane 5 of Figure 10 shows molecular weight (M_r) markers. Gels were stained with Coomassie Brilliant Blue, destained, and photographed or dried and exposed to X-ray film (Kodak XAR-5; Eastman Kodak Co., Rochester, NY) for autoradiography to visualize proteins.

As shown in Figure 10, the B7Ig fusion protein migrated during SDS-PAGE under nonreducing conditions predominantly as a single species of M_r 70,000, with a small amount of material migrating as a M_r approximately 150,000 species. After reduction, a single M_r approximately 75,000 species was observed. CD28Ig migrated as a M_r approximately 140,000 species under non-reducing conditions and a M_r approximately 70,000 species after reduction, indicating that it was expressed as a homodimer. Since the Ig C γ 1 hinge cysteines had been mutated, disulfide linkage probably involved cysteine residues which naturally form interchain bonds in the CD28 homodimer (Hansen et al., Immunogenetics 10:247 (1980)).

DNAs encoding the amino acid sequences corresponding to the B7Ig fusion protein and CD28Ig fusion protein have been deposited with the ATCC in Rockville, Maryland, under the terms of the Budapest Treaty on May 31, 1991 and there have been accorded accession numbers: 68627 (B7Ig) and 68628 (CD28Ig).

II. Characterization of B7Ig and CD28Ig Cy1 Fusion Proteins

To investigate the functional activities of B7Ig and CD28Ig, binding of CHO cell lines expressing CD28 or B7 was tested as follows. In early experiments, spent culture media from transfected COS cells was used as a source of fusion protein, while in later experiments, purified proteins were used (see Figure 11).

Binding of B7Ig and CD28Ig to CHO cells. Binding of CD28Ig and B7Ig fusion proteins was detected by addition of FITC-conjugated goat anti-human Ig second step reagent as described above. B7Ig was bound by CD28⁺ CHO, while CD28Ig was bound by B7⁺ CHO. B7Ig also bound weakly to B7⁺ CHO (Figure 11), suggesting that this molecule has a tendency to form homophilic interactions. No binding was detected of chimeric mAb L6 containing human Ig Cy1, or another fusion protein, CD5Ig. Thus B7Ig and CD28Ig retain binding activity for their respective counter-receptors.

The apparent affinity of interaction between B7 and CD28 was next determined. B7Ig was either iodinated or metabolically labeled with [³⁵S]methionine, and radiolabeled derivatives were tested for binding to immobilized CD28Ig or to CD28⁺ CHO cells.

Radiolabeling of B7Ig. Purified B7Ig (25 µg) in a volume of 0.25 ml of 0.12 M sodium phosphate, pH 6.8 was iodinated using 2 mCi ¹²⁵I and 10 µg of chloramin T. After 5 min at 23°C, the reaction was stopped by the addition of 20 µg sodium metabisulfite, followed by 3 mg of KI and 1 mg of BSA. Iodinated protein was separated from untreated ¹²⁵I by chromatography on a 5-ml column of Sephadex G-10 equilibrated with PBS containing 10% FCS. Peak fractions were collected

and pooled. The specific activity of ^{125}I -B7Ig labeled in this fashion was 1.5×10^6 cpm/pmol.

B7Ig was also metabolically labeled with
5 [^{35}S]methionine. COS cells were transfected with a plasmid encoding B7Ig as described above. At 24 h after transfection, [^{35}S]methionine (<800 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added to concentrations of 115 $\mu\text{Ci/ml}$ in DMEM containing 10% FCS and 10% normal levels of methionine.
10 After incubation at 37°C for 3 d, medium was collected and used for purification of B7Ig as described above. Concentrations of [^{35}S]methionine-labeled B7Ig were estimated by comparison of staining intensity after SDS-PAGE with intensities of known amounts of unlabeled B7Ig. The specific
15 activity of [^{35}S]methionine-labeled B7Ig was approximately 2×10^6 cpm/ μg .

Binding Assays. For assays using immobilized CD28Ig, 96-well plastic dishes were coated for 16-24 h with a solution
20 containing CD28Ig (0.5 μg in a volume of 0.05 ml of 10 mM Tris, pH 8). Wells were then blocked with binding buffer (DMEM containing 50 mM BES, pH 6.8, 0.1% BSA, and 10% FCS) (Sigma Chemical Co., St. Louis, MO) before addition of a solution (0.09 ml) containing ^{125}I -B7Ig (approximately 3×10^6
25 cpm, 2×10^6 cpm/pmol) or [^{35}S]-B7Ig (1.5×10^5 cpm) in the presence or absence of competitor to a concentration of 24 nM in the presence of the concentrations of unlabeled chimeric L6 mAb, mAb 9.3, mAb BB-1 or B7Ig, as indicated in Figure 12. After incubation for 2-3 h at 23°C, wells were washed once
30 with binding buffer, and four times with PBS. Plate-bound radioactivity was then solubilized by addition of 0.5 N NaOH, and quantified by liquid scintillation or gamma counting. In Figure 12, radioactivity is expressed as a percentage of radioactivity bound to wells treated without competitor (7,800

cpm). Each point represents the mean of duplicate determinations; replicates generally varied from the mean by $\leq 20\%$. Concentrations were calculated based on a M_r of 75,000 per binding site for mAbs and 51,000 per binding site for B7Ig. When binding of ^{125}I -B7 to CD28⁺ CHO cells was measured, cells were seeded (2.5×10^4 /well) in 96-well plates 16-24 h before the start of the experiment. Binding was otherwise measured as described above.

The results of a competition binding experiment using ^{125}I -B7Ig and immobilized CD28Ig are shown in Figure 12. Binding of ^{125}I -B7Ig was competed in dose-dependent fashion by unlabeled B7Ig, and by mAbs 9.3 and BB-1. mAb 9.3 was the most effective competitor (half-maximal inhibition at 4.3 nM), followed by mAb BB-1 (half-maximal inhibition at 140 nM) and B7 Ig (half-maximal inhibition at 280 nM). Thus, mAb 9.3 was approximately 65-fold more effective as a competitor than B7Ig, indicating that the mAb has greater apparent affinity for CD28. The same relative difference in avidities was seen when [^{35}S]methionine-labeled B7Ig was used. Chimeric mAb L6 did not significantly inhibit binding. The inhibition at high concentrations in Figure 12 was not seen in other experiments.

When the competition data shown in Figure 12 were replotted in the Scatchard representation (Figure 13), a single class of binding sites was observed (binding constant (K_d) estimated from the slope of the line best fitting the experimental data ($r = -0.985$), K_d of approximately 200 nM. An identical K_d was detected for binding of ^{125}I -B7Ig to CD28⁺ CHO cells. Thus, both membrane bound CD28 and immobilized CD28Ig showed similar apparent affinities for ^{125}I -B7.

Binding of B7Ig to CD28 expressed on T cells

Although B7Ig bound to immobilized CD28Ig, and to CD28⁺ CHO cells, it was not known whether B7Ig could bind to CD28 naturally expressed on T cells. This is an important consideration since the level of CD28 on transfected cells was approximately 10-fold higher than that found on PHA-activated T cells as shown above in Example 1. PHA-activated T cells were prepared as follows.

Cell separation and Stimulation. PBL were isolated by centrifugation through Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) and cultured in 96-well, flat-bottomed plates (4×10^4 cells/well, in a volume of 0.2 ml) in RPMI containing 10% FCS. Cellular proliferation of quadruplicate cultures was measured by uptake of [³H]thymidine during the last 5 h of a 3 day (d) culture. PHA-activated T cells were prepared by culturing PBL with 1 μ g/ml PHA (Wellcome) for 5 d, and 1 d in medium lacking PHA. Viable cells were collected by sedimentation through Lymphocyte Separation Medium before use.

PHA-activated T cells were then tested for binding of B7Ig (10 μ g/ml) by FACS^R analysis after indirect immunofluorescence as described above. Where indicated (Figure 14), mAbs 9.3 or BB-1 were also added at 10 μ g/ml to cells simultaneously with B7Ig. Bound mAb was detected with a FITC-conjugated goat anti-human Ig Gyl reagent.

As shown in Figure 14, these cells bound significant levels of B7Ig, and binding was inhibited by mAbs 9.3 and BB-1.

The identity of B7Ig-binding proteins was also determined by immunoprecipitation analysis of ^{125}I -surface labeled cells as follows.

5 Cell Surface Iodination and Immunoprecipitation. PHA-
activated T cells were cell-surface labeled with ^{125}I using
lactoperoxidase and H_2O_2 as described by Vitetta et al., J.
10 Exp. Med. 134:242 (1971), incorporated by reference herein.
Aliquots of a nonionic detergent extract of labeled cells
(approximately 3×10^8 cpm in a volume of 0.12 ml) were
prepared as described by Linsley et al., J. Biol. Chem.
15 263: 8390 (1988), incorporated by reference herein, and
subjected to immunoprecipitation analysis and SDS-PAGE, as
described above using a 5-15% acrylamide gradient, under
reducing (Figure 15, +BME, lanes 1-7) or non-reducing
conditions (-BME, lanes 8 and 9), with no addition (lane 1),
addition of mAb 9.3 (5 μg , lane 2), addition of B7Ig (10 μg ,
lane 3), or addition of chimeric L6 mAb (10 μg , lane 7).

20 As shown in Figure 15, Both mAb 9.3 and B7Ig
immunoprecipitated a protein having a M_r of approximately
45,000 under reducing conditions, and proteins having a M_r of
approximately 45,000 and approximately 90,000 under
nonreducing conditions, with the latter form being more
25 prominent. The protein having a M_r of approximately 45,000
found in the sample precipitated with chimeric mAb L6 was due
to spillover and was not observed in other experiments. mAb
9.3 was more effective at immunoprecipitation than B7Ig, in
agreement with the greater affinity of the mAb (Figures 12 and
30 13). Identical results were obtained when CD28⁺ CHO cells were
used for immunoprecipitation analysis. Preclearing of CD28 by
immunoprecipitation with mAb 9.3 also removed B7Ig-
precipitable material, indicating that both mAb 9.3 and B7Ig
bound the same ^{125}I -labeled protein.

Taken together, the results in these experiments indicate that CD28 is the major receptor for B7Ig on PHA-activated T cells.

5 Effects of B7 Binding to CD28 on CD28-mediated Adhesion

mAbs to CD28 have potent biological activities on T cells, suggesting that interaction of CD28 with its natural ligand(s) may also have important functional consequences. As
10 a first step in determining functional consequences of interaction between B7 and CD28, it was determined whether B7Ig could block the CD28-mediated adhesion assay described above. The adhesion of ⁵¹Cr-labeled PM lymphoblastoid cells to monolayers of CD28⁺ CHO cells was measured as described above,
15 in the presence of the indicated amounts of mAb 9.3 or B7Ig. Data are expressed in Figure 16 as a percentage of cells bound in the absence of competitor (40,000 cpm or approximately 1.1 X 10⁵ cells). Each point represents the mean of triplicate determinations; coefficients of variation were ≤ 25%.

As shown in Figure 16, B7Ig blocked CD28-mediated adhesion somewhat less effectively than mAb 9.3 (half-maximal inhibition at 200 nM as compared with 10 nM for mAb 9.3). The relative effectiveness of these molecules at inhibiting CD28-
25 mediated adhesion was similar to their relative binding affinities in competition binding experiments (Figure 12). CD28Ig failed to inhibit CD28-mediated adhesion at concentrations of up to 950 nM, suggesting that much higher levels of CD28Ig were required to compete with the high local
30 concentrations of CD28 present on transfected cells.

The Effects of B7 On T Cell Proliferation

It was further investigated whether triggering of
35 CD28 by B7 was costimulatory for T cell proliferation. The

ability of B7Ig to costimulate proliferation of PBL together with anti-CD3 was first explored. PBL were isolated and cultured in the presence of the costimulators of T cell proliferation indicated in Table 2. Anti-CD3 stimulation was with mAb G19-4 at 1 μ g/ml in solution. For CD28 stimulation, mAb 9.3 or B7Ig were added in solution at 1 μ g/ml, or after immobilization on the culture wells by pre-incubation of proteins at 10 μ g/ml in PBS for 3 h at 23°C and then washing the culture wells. B7⁺ CHO and control dhfr⁺ CHO cells were irradiated with 1,000 rad before mixing with PBL at a 4:1 ratio of PBL/CHO cells. After culture for 3 d, proliferation was measured by uptake of [³H] thymidine for 5 h. Values shown are means of determinations from quadruplicate cultures (SEM < 15%).

In several experiments, B7Ig in solution at concentrations of 1-10 μ g/ml showed only a modest enhancement of proliferation even though the anti-CD28 mAb 9.3 was effective. Because CD28 crosslinking has been identified as an important determinant of CD28 signal transduction (Ledbetter et al., Blood 75:1531 (1990)), B7Ig was also compared to 9.3 when immobilized on plastic wells (Table 2, Exp. 1).

Table 2

		<u>[³H]-T incorporation</u>	
<u>Exp. 1</u>	<u>CD28 Stimulation</u>	<u>-Anti-CD3</u>	<u>+Anti-CD3</u>
cpm X 10 ⁻³			
1	None	0.1	26.0
	mAb 9.3 (soln.)	0.3	156.1
	mAb 9.3 (immob.)	0.1	137.4
	B7Ig (immob.)	0.1	174.5
2	None	0.2	19.3
	mAb 9.3 (soln.)	0.4	75.8
	B7 + CHO cells	9.4	113.9
	dhfr + CHO cells	23.8	22.1

Under these conditions, B7Ig was able to enhance proliferation and compared favorably with mAb 9.3. B7⁺ CHO cells also were tested and compared with control dhfr⁺ CHO cells for costimulatory activity on resting lymphocytes (Table 2, Exp. 2). In this experiment, proliferation was seen with dhfr⁺ CHO cells in the absence of anti-CD3 mAb because of residual incorporation of [³H]thymidine after irradiation of these cells. The stimulation by dhfr⁺ cells was not enhanced by anti-CD3 mAb and was not observed in other experiments (Tables 3 and 4) where transfected CHO cells were added at lower ratios.

For the experiments shown in Table 3, PHA blasts were cultured at 50,000 cells/well with varying amounts of irradiated CH) cell transfectants. After 2 d of culture, proliferation was measured by a 5 h pulse of [³H]thymidine. Shown are means of quadruplicate determinations (SEM < 15%). Background proliferation of PHA blasts without added CHO cells was 11,200 cpm. [³H]thymidine incorporation by irradiated B7⁺ CHO and CD5⁺ CHO cells alone was > 1,800 cpm at each cell concentration and was subtracted from the values shown. For the experiments summarized in Table 4, PHA blasts were stimulated as described in Table 3, with irradiated CHO cells at a ratio of 40:1 T cells/CHO cells. mAbs were added at 10 µg/ml at the beginning of culture. mAb LB-1 (Yokochi et al., supra) is an isotype-matched control for mAb BB-1. Proliferation was measured by uptake of [³H]thymidine during a 5 h pulse after 2 d of culture. Values represent means of quadruplicate cultures (SEM <15%).

B7⁺ CHO cells were very effective at costimulation with anti-CD3 mAb, indicating that cell surface B7 had similar activity in this assay as the anti-CD28 mAbs.

B7⁺ CHO cells were also tested as to whether they could directly stimulate proliferation of resting PHA blasts which respond directly to CD28 crosslinking by mAb 9.3. Again, the B7⁺ CHO cells were very potent in stimulating proliferation (Table 3) and were able to do so at very low cell numbers (PHA blast:B7⁺ CHO ratios of >800:1). The control CD5⁺ CHO cells did not possess a similar activity. (In a number of different experiments neither dhfr CHO, CD5⁺ CHO, nor CD7⁺ CHO cells stimulated T cell proliferation. These were therefore used interchangeably as negative controls for effects induced by B7⁺ CHO cells. The stimulatory activity of B7⁺ CHO was further shown to result from CD28/B7 interaction, since mAb BB1 inhibited stimulation by the B7⁺ CHO cells without affecting background proliferation in the presence of CD7⁺ CHO cells (Table 4). mAb LB-1 (Yokochi et al., *supra*), an IgM mAb to a different B cell antigen, did not inhibit proliferation. mAb 9.3 (Fab fragments) inhibited proliferation induced by B7⁺ CHO and as well as background proliferation seen with CD7⁺ CHO cells.

Table 3

[³H]-T incorporation

<u>T cells/CHO cells</u>	<u>+ B7⁺ CHO</u>	<u>+ CD5⁺ CHO</u>
	<u>cpm X 10⁻³</u>	
25:1	92.7	15.5
50:1	135.4	19.4
100:1	104.8	16.8
200:1	90.3	17.7
400:1	57.0	13.7
800:1	42.3	17.6

Table 4

	Stimulation	mAb	[³ H]-T incorporation
5			cpm X 10 ⁻³
	None	None	10.8
	B7 ⁺ CHO	None	180
	B7 ⁺ CHO	9.3 Fab	132
10	B7 ⁺ CHO	BB-1	98.3
	B7 ⁺ CHO	LB-1	196
	CD7 ⁺ CHO	None	11.5
	CD7 ⁺ CHO	9.3 Fab	10.0
15	CD7 ⁺ CHO	BB-1	10.0
	CD7 ⁺ CHO	LB-1	11.3

20 These experiments show that B7 is able to stimulate signal transduction and augment T cell activity by binding to CD28, but that crosslinking is required and B7 expressed on the cell surface is most effective.

25 The Effects of B7 on IL-2 mRNA Accumulation

Effects of CD28/B7 interactions on IL-2 production were investigated by analyzing transcript levels in PHA-blasts stimulated with B7⁺ CHO cells or CD7⁺ CHO cells. RNA was
30 prepared from stimulated cells and tested by RNA blot analysis for the presence of IL-2 transcripts as follows.

PHA blasts (5 X 10⁷) were mixed with transfected CHO cells at a ratio of 40:1 T cells/CHO cells, and/or mAbs as
35 indicated in Figure 17. mAb 9.3 was used at 10 µg/ml. mAb BB-1 was added at 20 µg/ml 1 h before addition of B7⁺ CHO cells. When mAb 9.3 was crosslinked, goat anti-mouse Ig (40 µg/ml) was added 10 min after addition of mAb 9.3. Cells were incubated for 6 h at 37°C and RNA was isolated and subjected to
40 RNA blot analysis using ³²P-labeled IL-2 or GAPDH probes as described below.

RNA was prepared from stimulated PHA blasts by the procedure described by Chomczynski and Sacchi, Anal. Biochem 162:156 (1987), incorporated by reference herein. Aliquots of RNA (20 μ g) were fractionated on formaldehyde agarose gels and then transferred to nitrocellulose by capillary action. RNA was crosslinked to the membrane by UV light in a Stratalinker (Stratagene, San Diego, CA), and the blot was prehybridized and hybridized with a 32 P-labeled probe for human IL-2 (prepared from an approximately 600-bp cDNA fragment provided by Dr. S. Gillis; Immunex Corp., Seattle, WA). Equal loading of RNA samples was verified both by rRNA staining and by hybridization with a rat glyceraldehyde-6-phosphate dehydrogenase probe (GAPDH, an approximately 1.2-kb cDNA fragment provided by Dr. A. Purchio, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA).

As shown in Figure 17, B7⁺ CHO cells, but not CD7⁺ CHO cells, induced accumulation of IL-2 mRNA transcripts. Induction by B7⁺ CHO cells was partially blocked by mAb BB-1. Induction by B7⁺ CHO cells was slightly better than achieved by mAb 9.3 in solution, but less effective than mAb 9.3 after crosslinking with goat anti-mouse Ig. Thus, triggering of CD28 by cell surface B7 on apposing cells stimulated IL-2 mRNA accumulation.

The apparent K_d value for the interaction of soluble Ig Cy fusions of CD28 and B7 (approximately 200 nM), obtained from the above experiments, is within the range of affinities observed for mAbs (2-10,000 nM; Alzari et al., Annu. Ref. Immunol. 6:555 (1988)) and compares favorably with the affinities estimated for other lymphoid adhesion molecules. Schneck et al., (Cell 56:47 (1989)) estimated the affinity (K_d approximately 100 nM) between a murine T cell hybridoma TCR and soluble alloantigen (class I MHC molecules). A K_d of 400 nM was measured between CD2 and LFA3 (Recny et al., J. Biol.

Chem. 265:8542 (1990)). The affinity of CD4 for class II MHC, while not measured directly, was estimated (Clayton et al., Nature (Lond.) 339:548 (1989)) to be $\geq 10,000$ times lower than the affinity of gp120-CD4 interactions ($K_d = 4$ nM; Lasky et al., Cell 50:975 (1987)). Thus, the affinity of B7 for CD28 appears greater than affinities reported for some other lymphoid adhesion systems.

The degree to which the apparent K_d of CD28/B7 interaction reflects their true affinity, as opposed to their avidity, depends on the valency and/or aggregation of the fusion protein preparations. The degree of aggregation of these preparations was examined by size fractionation (TSK G3000SW column eluted with PBS). Under these conditions, B7Ig eluted at M_r approximately 350,000, and CD28Ig at M_r approximately 300,000. Both proteins thus behaved in solution as larger molecules than they appeared by SDS-PAGE (Figure 10), suggesting that they may form higher aggregates. Alternatively, these results may indicate that both fusion proteins assume extended conformations in solution, resulting in large Stokes radii. Regardless, the interaction that was measured using soluble proteins probably underestimates the true avidity between CD28 and B7 in their native membrane-associated state.

The relative contribution of different adhesion systems to the overall strength of T cell-B cell interactions is not easily gauged, but is likely a function of both affinity/avidity and the densities on apposing cell surfaces of the different receptors and counter-receptors involved. Since both CD28 and B7 are found at relatively low levels on resting lymphoid cells (Lesslauer et al., Eur. J. Immuno. 16:1289 (1986); Freeman et al., supra 1989), they may be less involved than other adhesion systems (Springer Nature (Lond.) 346:425 (1990)) in initiating intercellular interactions. The

primary role of CD28/B7 interactions may be to maintain or amplify a response subsequent to induction of these counter-receptors on their respective cell types.

5 Binding of B7 to CD28 on T cells was costimulatory for T cell proliferation (Tables 2-4) suggesting that some of the biological effects of anti-CD28 mAbs result from their ability to mimic T cell activation resulting from natural interaction between CD28 and its counter-receptor, B7. mAb
10 9.3 has greater affinity for CD28 than does B7Ig (Figures 15 and 16), which may account for the extremely potent biological effects of this mAb (June et al., supra 1989) in costimulating polyclonal T cell responses. Surprisingly, however, anti-CD28 mAbs are inhibitory for antigen-specific T cell responses
15 (Damle et al., Proc. Natl. Acad. Sci. USA 78:5096 (1981); Lesslauer et al., supra 1986). This may indicate that antigen-specific T cell responses are dependent upon costimulation via CD28/B7 interactions, and that inhibition therefore results from blocking of CD28 stimulation. Despite
20 the inhibition, CD28 must be bound by mAb under these conditions, implying that triggering by mAb is not always equivalent to triggering by B7. Although mAb 9.3 has higher apparent affinity for CD28 than B7 (Figure 12), it may be unable under these circumstances to induce the optimal degree
25 of CD28 clustering (Ledbetter et al., supra 1990) for simulation.

CD28/B7 interactions may also be important for B cell activation and/or differentiation. As described above in
30 Example 2, mAbs 9.3 and BB-1 block T_h cell-induced Ig production by B cells. This blocking effect may be due in part to inhibition by these mAbs of production of T_h -derived B cell-directed cytokines, but may also involve inhibition of B cell activation by interfering with direct signal transduction via
35 B7. These results suggest that cognate activation of B

lymphocytes, as well as T_h lymphocytes, is dependent upon interaction between CD28 and B7.

5 The above results demonstrate that the ligand for
CD28 receptor, the B7 antigen, is expressed on activated B
cells and cells of other lineages. These results also show
that CD28 receptor and its ligand, B7, play a pivotal role
during both the activation of $CD4^+$ T_h cell and T_h -induced
differentiation of B cells. The inhibition of anti-CD28 and
10 anti-B7 mAbs on the cognate T_h :B interaction also provide the
basis for employing the CD28Ig and B7Ig fusion proteins, and
monoclonal antibodies reactive with these proteins, to treat
various autoimmune disorders associated with exaggerated B cell
activation such as insulin-dependent diabetes mellitus,
15 myasthenia gravis, rheumatoid arthritis and systemic lupus
erythematosus (SLE).

As will be apparent to those skilled in the art to
which the invention pertains, the present invention may be
20 embodied in forms other than those specifically disclosed
above without departing from the spirit or essential
characteristics of the invention. The particular embodiments
of the invention described above, are, therefore, to be
considered as illustrative and not restrictive. The scope of
25 the present invention is as set forth in the appended claims
rather than being limited to the examples contained in the
foregoing description.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT: Linsley, Peter S
Ledbetter, Jeffrey A
Damle, Nitin K
Brady, William

10

(ii) TITLE OF INVENTION: LIGAND FOR CD28 RECEPTOR ON B
CELLS AND METHODS

(iii) NUMBER OF SEQUENCES: 7

15

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY: Pasadena
(D) STATE: California
(E) COUNTRY: United States
(F) ZIP: 91101

20

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

25

30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

35

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Mandel, SaraLynn
- (B) REGISTRATION NUMBER: 31,853
- (C) REFERENCE/DOCKET NUMBER: 7794

5

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (818) 796-4000
- (B) TELEFAX: (818) 795-6321

10 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

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- (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid

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(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(A) LENGTH: 84 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

20 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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25 CCGTCCCCAG CACCTGAACT CCTG 84

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(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

10

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41

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

25

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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47

35

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- (A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCCACAGTG CAGTGATCAT TTGGATCCTG GCATGTGAC

39

We claim:

1. A method for regulating functional T cell responses
5 comprising contacting CD28 positive T cells with a ligand for CD28 receptor.
2. The method of claim 1 wherein said ligand is B7 antigen.
- 10 3. The method of claim 2 wherein said T cells are contacted with a fragment or derivative of said B7 antigen.
4. The method of claim 3 wherein said fragment or derivative contains at least a portion of the extracellular domain of the
15 B7 antigen.
5. The method of claim 4 wherein said fragment is a polypeptide having an amino acid sequence containing amino acid residues from about position 1 to about position 215 of
20 the amino acid sequence corresponding to the extracellular domain of B7 antigen.
6. The method of claim 4 wherein said derivative comprises a fusion polypeptide having a first amino acid sequence
25 corresponding to the extracellular domain of B7 antigen and a second amino acid sequence corresponding to a moiety that alters the solubility, affinity and/or valency of said B7 antigen for binding to the CD28 receptor.
- 30 7. The method of claim 6 wherein said moiety is an immunoglobulin constant region.

8. The method of claim 6 wherein said derivative comprises a fusion polypeptide having a first amino acid sequence containing amino acid residues from about position 1 to about position 215 of the amino acid sequence corresponding to the extracellular domain of B7 antigen and a second amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin Cy1.

9. The method of claim 1 wherein said B7 antigen is immobilized to crosslink CD28 receptor on said T cells.

10. The method of claim 9 wherein said T cells are reacted with CHO cells expressing B7 antigen.

11. B7Ig fusion protein reactive with the CD28 receptor on T cells comprising a polypeptide having a first amino acid sequence containing amino acid residues from about position 1 to about position 215 of the amino acid sequence encoding the extracellular domain of B7 antigen and a second amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin Cy1.

12. B7Ig fusion protein corresponding to the amino acid sequence encoded by DNA having ATCC No. 68627.

13. The method of claim 1 wherein said B7 antigen is administered in vivo and further comprising administering a cytokine.

14. The method of claim 13 wherein said cytokine is selected from the group consisting of interleukins, interferons, transforming growth factors, tumor necrosis factors and colony stimulating factors.

15. The method of claim 1 further comprising adding anti-CD antibody to co-react with said T cells.

5 16. The method of claim 15 wherein said anti-CD antibody is anti-CD2 or anti-CD3 monoclonal antibody.

10 17. The method of claim 1 wherein said T cells are reacted with B cells expressing B7 antigen and said T cell responses are stimulated.

18. The method of claim 1 wherein said T cells are reacted with the ligand in soluble form and said T cell responses are inhibited.

15 19. A method for regulating functional T cell responses comprising reacting B7 positive cells with a ligand reactive with B7 antigen.

20 20. The method of claim 19 wherein said ligand reactive with B7 antigen is soluble and the interaction of said B7 positive cells with said T cells is inhibited.

25 21. The method of claim 19 wherein said ligand is a Fab fragment of a monoclonal antibody reactive with B7 antigen. and said T cell responses are inhibited.

22. The method of claim 21 wherein said monoclonal antibody is mAb BB-1.

23. The method of claim 21 wherein said monoclonal antibody is reactive with a fusion protein comprising a polypeptide having a first amino acid sequence containing amino acid residues from about position 1 to about position 215 of the amino acid sequence corresponding to the extracellular domain of B7 antigen and a second amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin Cγ1.

24. The method of claim 23 wherein said fusion protein is B7Ig corresponding to the amino acid sequence encoded by DNA having ATCC No. 68627.

25. A monoclonal antibody reactive with a fusion protein comprising a polypeptide having a first amino acid sequence containing amino acid residues from about position 1 to about position 215 of the amino acid sequence corresponding to the extracellular domain of B7 antigen and a second amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin Cγ1.

26. The method of claim 19 wherein said ligand is CD28 receptor and said T cell responses are inhibited.

27. The method of claim 26 wherein said ligand is a fragment or derivative of CD28 receptor.

28. The method of claim 27 wherein said fragment or derivative contains at least a portion of the extracellular domain of the CD28 receptor.

29. The method of claim 27 wherein said fragment is a polypeptide having an amino acid sequence containing amino acid residues from about position 1 to about position 134 of the amino acid sequence corresponding to the extracellular domain of CD28 receptor.

30. The method of claim 27 wherein said derivative comprises a fusion polypeptide having a first amino acid sequence corresponding to the extracellular domain of CD28 receptor and a second amino acid sequence corresponding to a moiety that alters the solubility, affinity and/or valency of said CD28 receptor for binding to B7 antigen.

31. The method of claim 30 wherein said moiety is an immunoglobulin constant region.

32. The method of claim 27 wherein said derivative is a CD28 fusion protein comprising a polypeptide having a first amino acid sequence containing amino acid residues from about position 1 to about position 134 of the amino acid sequence corresponding to the extracellular domain of CD28 receptor and a second amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin C γ 1.

33. CD28Ig fusion protein reactive with B7 antigen comprising a polypeptide having a first amino acid sequence containing amino acid residues from about position 1 to about position 134 of the amino acid sequence corresponding to the extracellular domain of CD28 receptor and a second amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin C γ 1.

34. CD28Ig fusion protein corresponding to the amino acid sequence encoded by DNA having ATCC No. 68628.

35. A method for inhibiting functional T cell responses comprising contacting CD28 positive T cells with a ligand reactive with CD28 receptor to prevent binding of said receptor to B7 antigen.

36. The method of claim 35 wherein said ligand is an anti-CD28 monoclonal antibody.

5 37. The method of claim 36 wherein said ligand is a Fab fragment of anti-CD28 monoclonal antibody.

38. The method of claim 36 wherein said antibody is 9.3 monoclonal antibody produced by hybridoma ATCC No. HB10271.

10 39. The method of claim 36 wherein said anti-CD28 antibody is reactive with a fusion protein comprising a polypeptide having a first amino acid sequence containing amino acid residues from about position 1 to about position 134 of the amino acid sequence corresponding to the extracellular domain of CD28
15 receptor and a second amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin C γ 1.

20 40. The method of claim 39 wherein said fusion protein is CD28Ig fusion protein corresponding to the amino acid sequence encoded by DNA having ATCC No. 68628.

25 41. The method of claim 35 wherein said ligand reactive with CD28 receptor is B7 antigen or a fragment or derivative of B7 antigen.

42. The method of claim 41 wherein said derivative is a B7Ig fusion protein.

30 43. A monoclonal antibody reactive with a fusion protein comprising a polypeptide having a first amino acid sequence containing amino acid residues from about position 1 to about position 134 of the amino acid sequence corresponding to the extracellular domain of CD28 receptor and a second amino acid sequence corresponding to the hinge, CH2 and CH3 regions of
35 human immunoglobulin C γ 1.

44. The monoclonal antibody of claim 43 reactive with CD28Ig having ATCC No. 68628.

5 45. A method for regulating the level of cytokines in vivo comprising administering to a subject a ligand reactive with CD28 receptor to bind to said CD28 receptor and inhibit the production of cytokines by said T cells.

10 46. The method of claim 45 wherein said ligand is B7 antigen.

47. The method of claim 45 wherein said ligand contains a portion of the extracellular domain of the B7 antigen.

15 48. The method of claim 47 wherein said ligand is a soluble B7Ig fusion protein.

20 49. The method of claim 48 wherein said B7Ig fusion protein is B7Ig corresponding to the amino acid sequence encoded by DNA having ATCC No. 68627.

50. The method of claim 45 wherein said ligand is a Fab fragment of anti-CD28 monoclonal antibody.

25 51. The method of claim 45 wherein said cytokines are selected from the group consisting of interleukins, interferons, transforming growth factors, tumor necrosis factor and colony stimulating factors.

30 52. A method for treating immune system diseases mediated by CD28 positive T cell interactions with B7 positive cells comprising administering to a subject a ligand for CD28 receptor to regulate the functional T cell response and/or to regulate cytokine levels.

35 53. The method of claim 52 wherein said ligand is B7 antigen.

54. The method of claim 52 wherein said ligand is soluble B7Ig fusion protein and said functional T cell response is inhibited.

5 55. The method of claim 52 wherein said ligand is anti-CD28 monoclonal antibody and said functional T cell response is inhibited.

10 56. The method of claim 52 wherein said ligand aggregates said CD28 receptor and said functional T cell response is stimulated.

15 57. The method of claim 56 wherein said ligand is immobilized B7 antigen.

20 58. The method of claim 52 wherein said cytokine is selected from the group consisting of interleukins, interferons, tumor growth factors, tumor necrosis factors and colony stimulating factors.

25 59. A method for treating cancer associated with expression of B7 antigen in vivo comprising administering to a subject ligand reactive with B7 antigen.

30 60. The method of claim 59 wherein said ligand is selected from the group consisting of anti-B7 monoclonal antibody, CD28 antigen and CD28Ig fusion protein.

61. The method of claim 59 wherein said cancer is B7 lymphoma.

62. The method of claim 59 wherein said cancer is T cell leukemia.

63. A method for inhibiting T cell proliferation in graft versus host disease comprising contacting T cells with a ligand for CD28 receptor and an immunosuppressant.

5 64. The method of claim 63 wherein said ligand for CD28 receptor is soluble B7 antigen.

65. The method of claim 63 wherein said ligand for CD28 receptor is soluble B7Ig fusion protein.

10

66. The method of claim 63 wherein said immunosuppressant is cyclosporine.

15

67. An assay method to detect a ligand reactive with a target receptor mediating cellular adhesion system comprising:

a) labeling test cells suspected of expressing ligand for a target receptor to form labeled test cells;

20

b) contacting said labeled test cells with cells expressing target receptor in a medium lacking divalent cations; and

25

c) determining whether the labeled test cells bind to said cells expressing target receptor,

whereby the presence of ligand reactive with said target receptor is detected.

30

68. The assay method of claim 67 wherein said target receptor is a receptor on lymphocytes.

69. The assay method of claim 68 wherein said target receptor is a receptor on T cells.

35

70. The assay method of claim 69 wherein the target receptor is CD28 and the ligand is B7 antigen.

5 71. The assay method of claim 67 wherein said target receptor is a receptor on B cells.

10 72. The assay method of claim 67 wherein said medium contains a divalent cation depletion reagent selected from the group consisting of EDTA and EGTA.

73. The assay method of claim 72 further comprising the step of fixing said cells expressing target receptor prior to addition of said reagent for depleting divalent cations.

15 74. The assay method of claim 73 wherein said step of fixing is carried out using paraformaldehyde.

20 75. The assay method of claim 67 wherein said cells expressing target receptor are grown in a monolayer prior to adding said test cells.

25 76. The assay method of claim 67 wherein said test cells are B cells and said cells expressing target receptor are chinese hamster ovary cells.

ABSTRACT OF THE INVENTION

5 The invention identifies the B7 antigen as a ligand
that is reactive with the CD28 receptor on T cells. Fragments
and derivatives of the B7 antigen and CD28 receptor, including
fusion proteins having amino acid sequences corresponding to
the extracellular domains of B7 or CD28 joined to amino acid
sequences encoding portions of human immunoglobulin C_γ1, are
10 described. Methods are provided for using B7 antigen, its
fragments and derivatives, and the CD28 receptor, its
fragments and derivatives, as well as antibodies and other
molecules reactive with B7 antigen and/or the CD28 receptor,
to regulate CD28 positive T cell responses, and immune
15 responses mediated by T cells. The invention also includes an
assay method for detecting ligands reactive with cellular
receptors mediating intercellular adhesion.

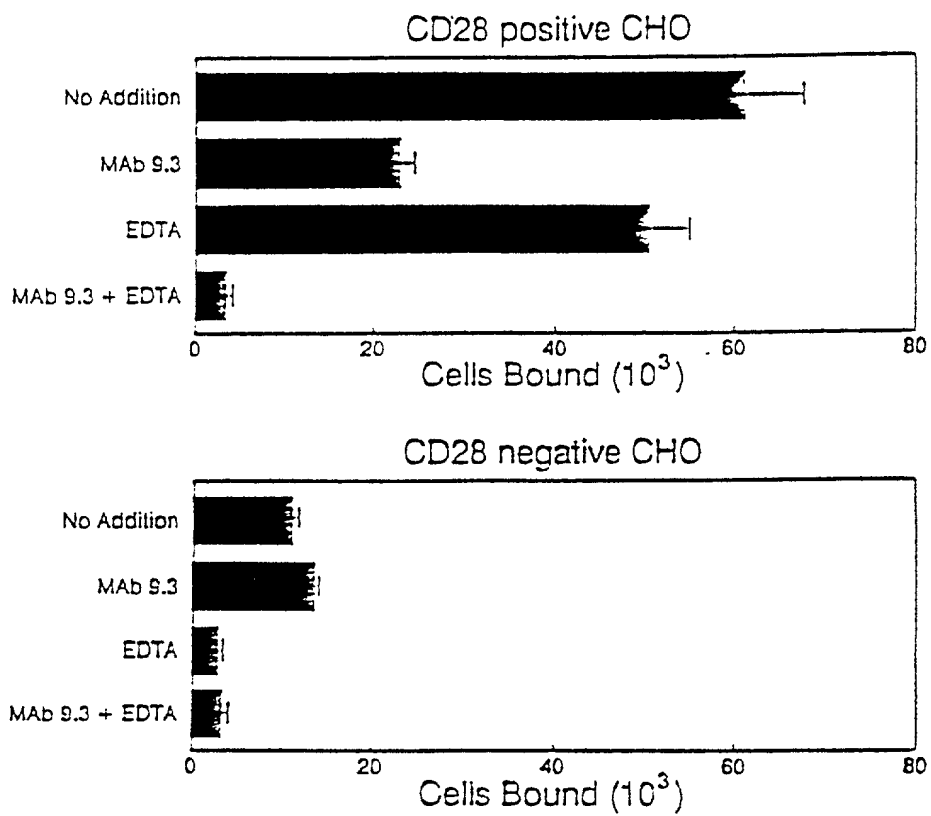


FIGURE 1

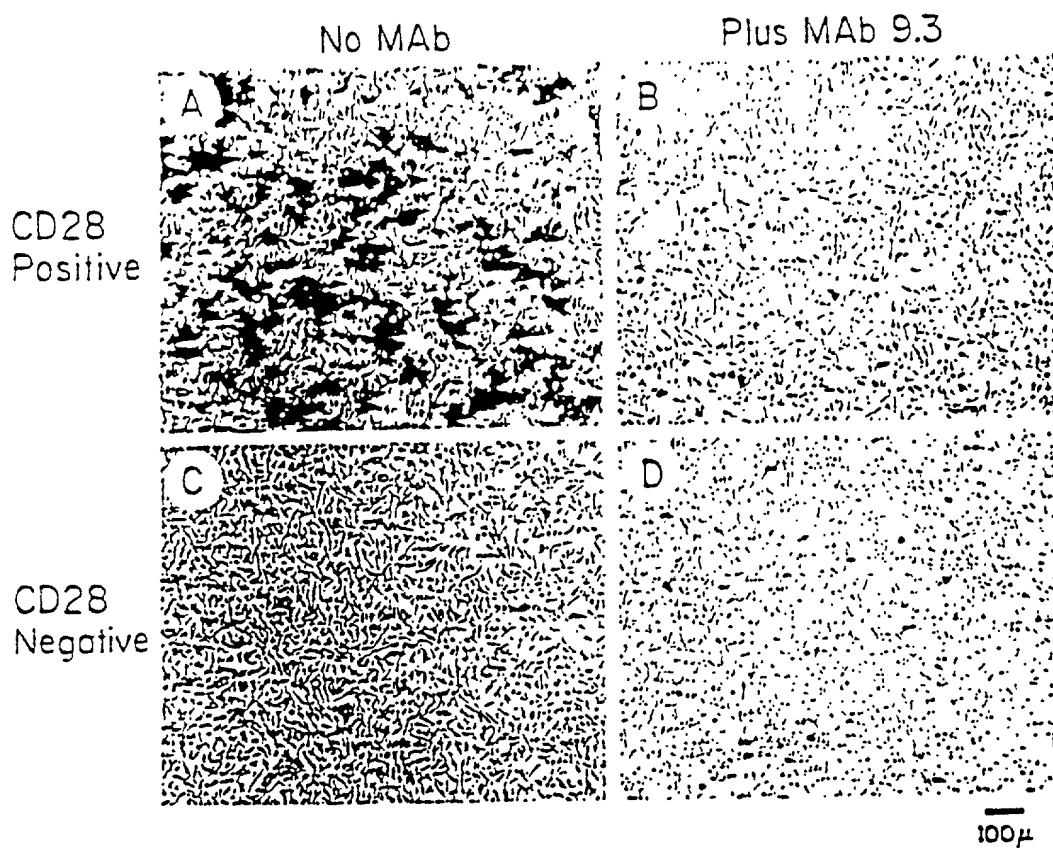


FIGURE 2

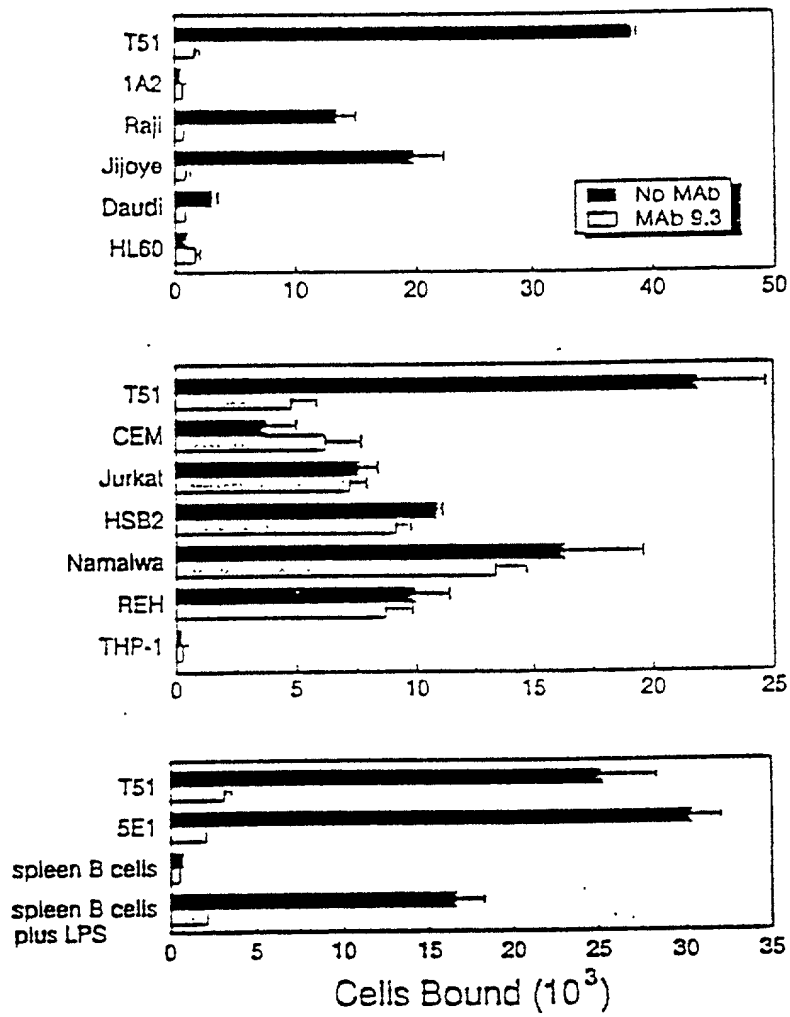


FIGURE 3

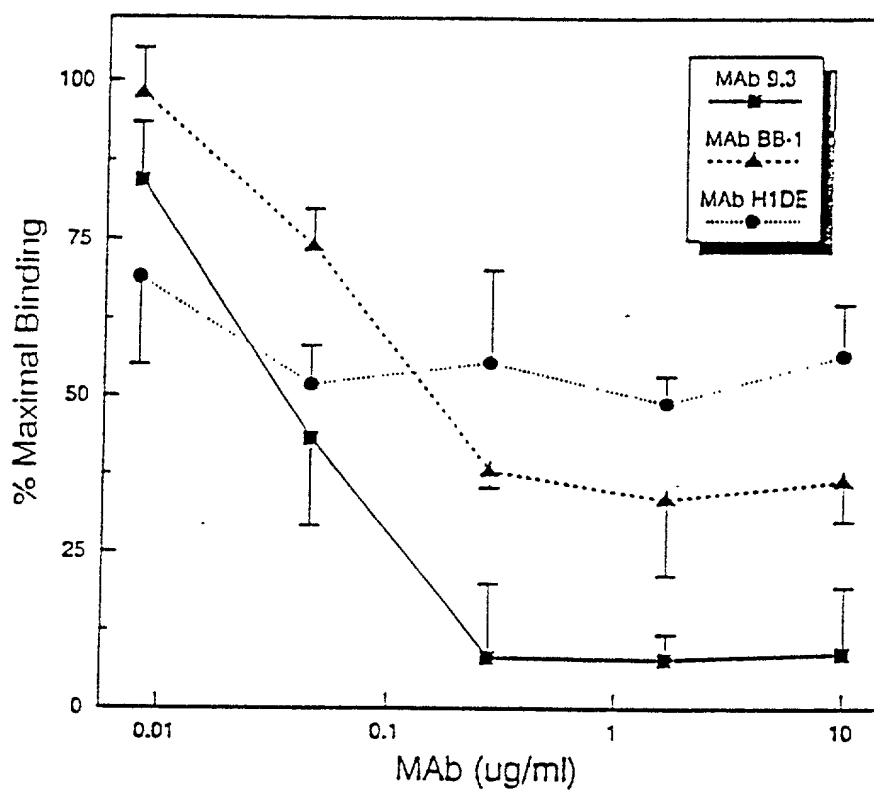


FIGURE 4

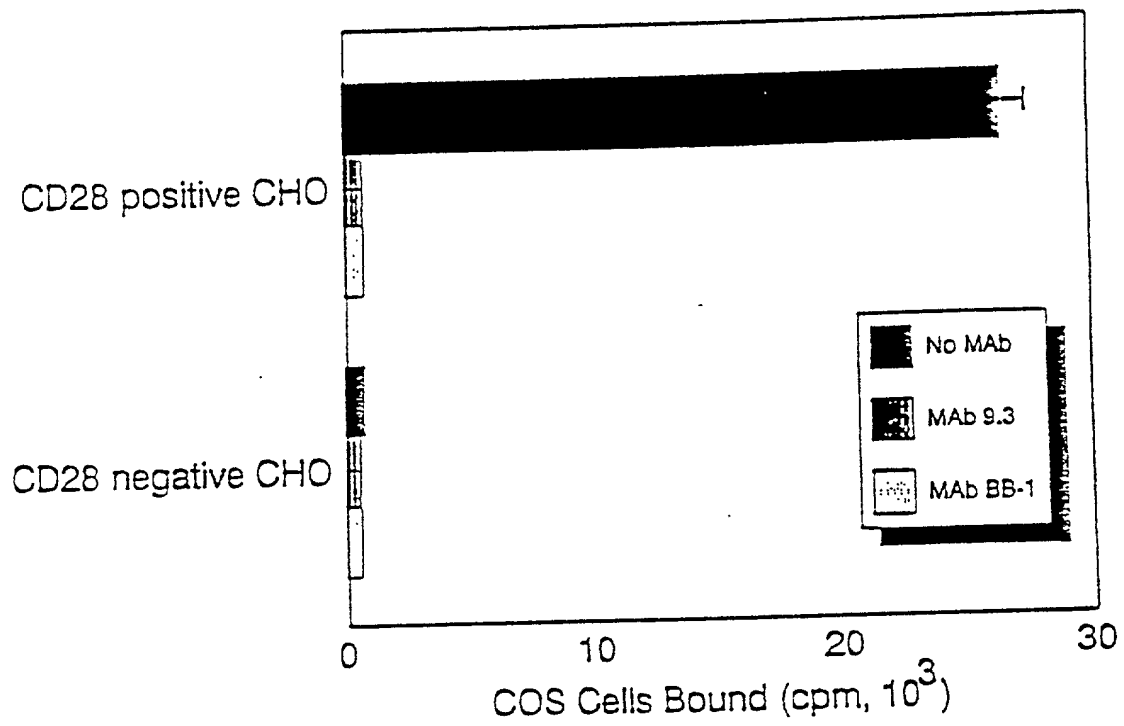


FIGURE 5

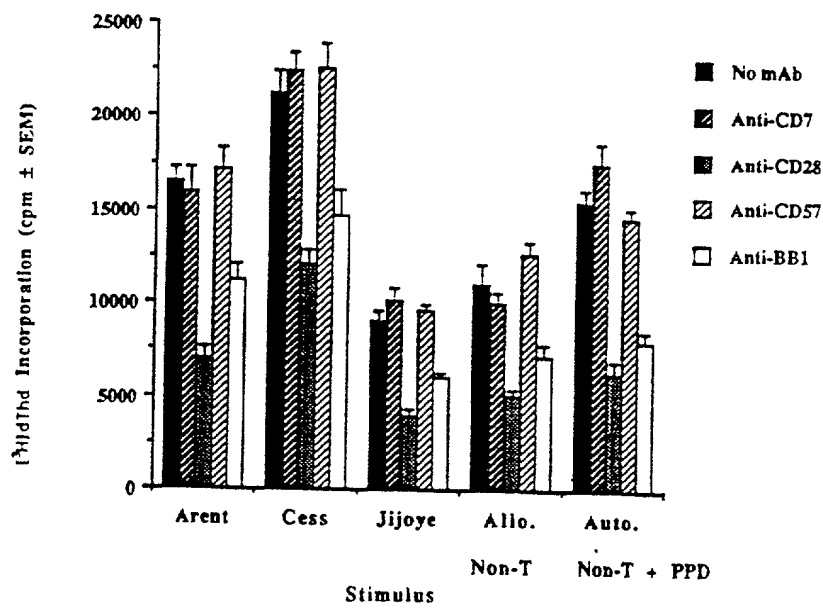
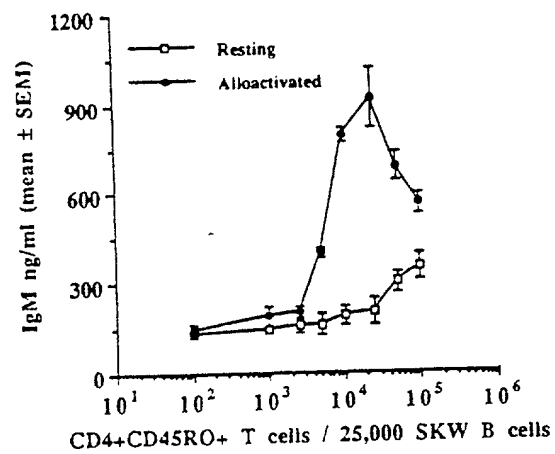


FIGURE 6

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7a



7b

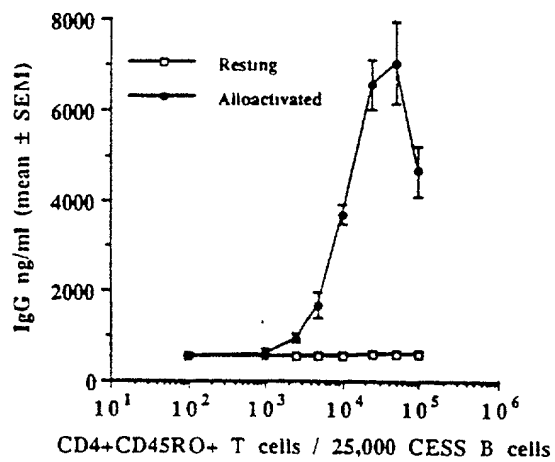


FIGURE 7

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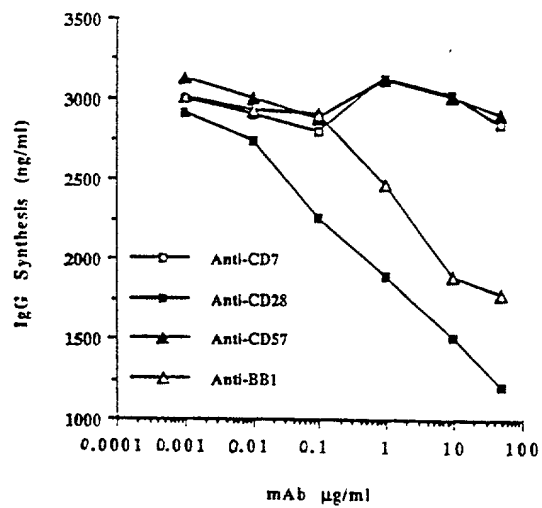
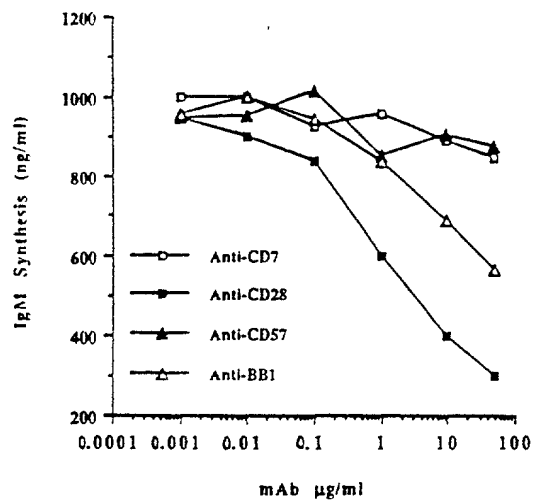


FIGURE 8

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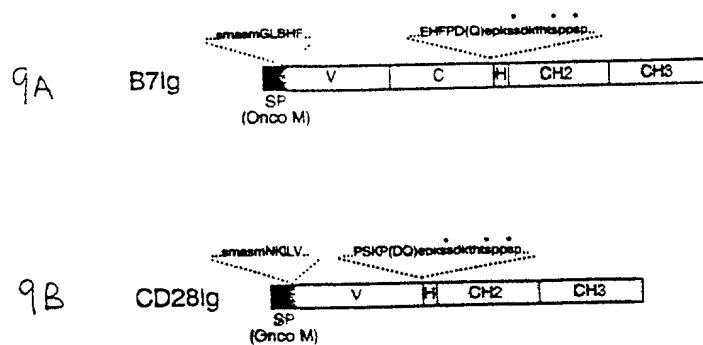


FIGURE 9

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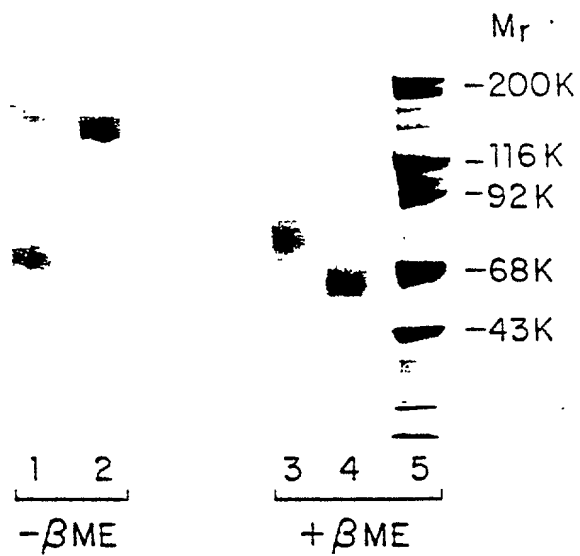


FIGURE 10

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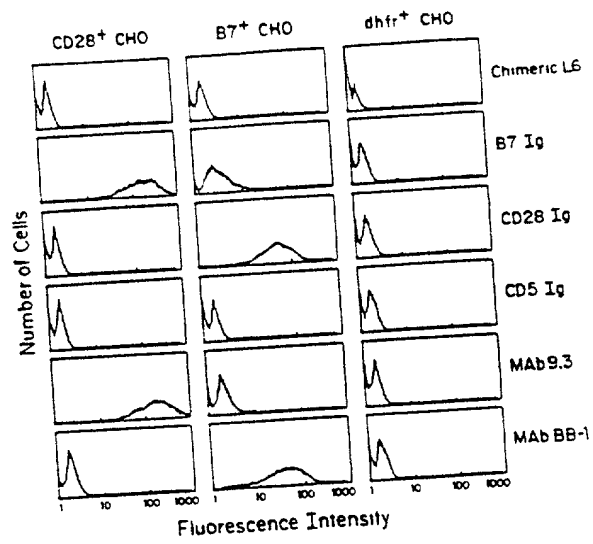


FIGURE 11

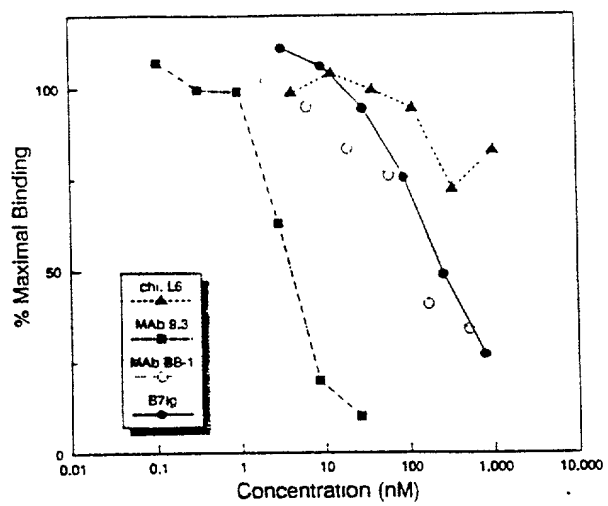


FIGURE 12

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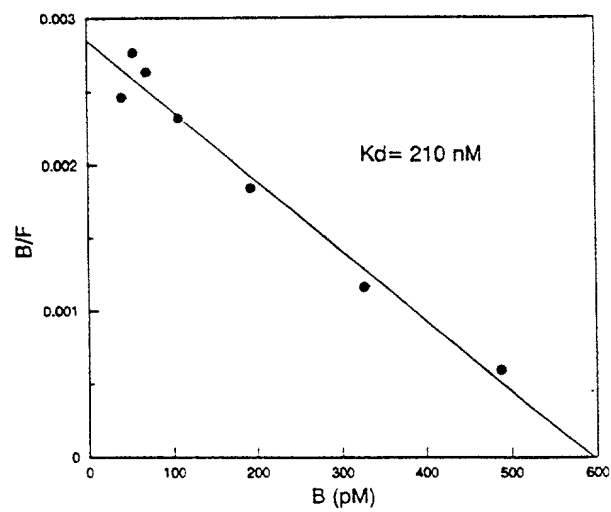


FIGURE 13

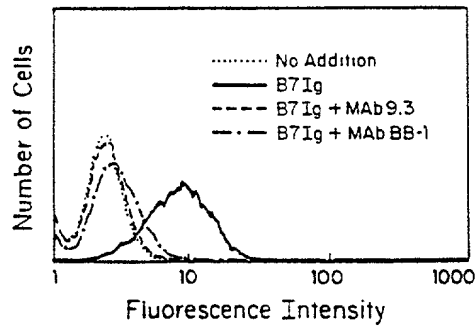


FIGURE 14

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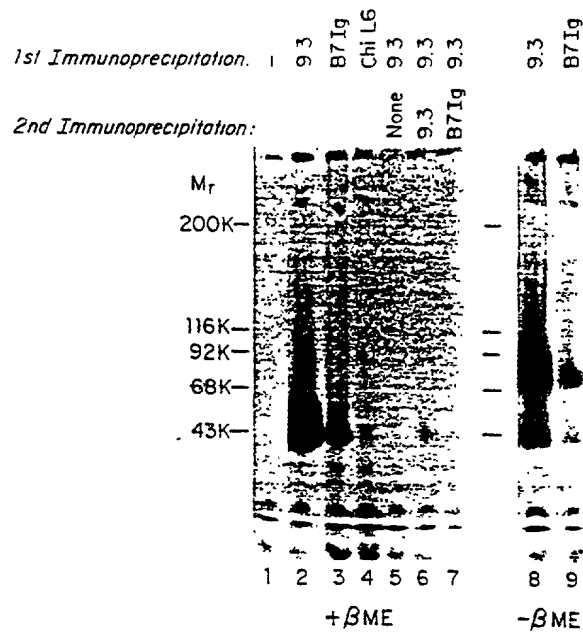


FIGURE 15

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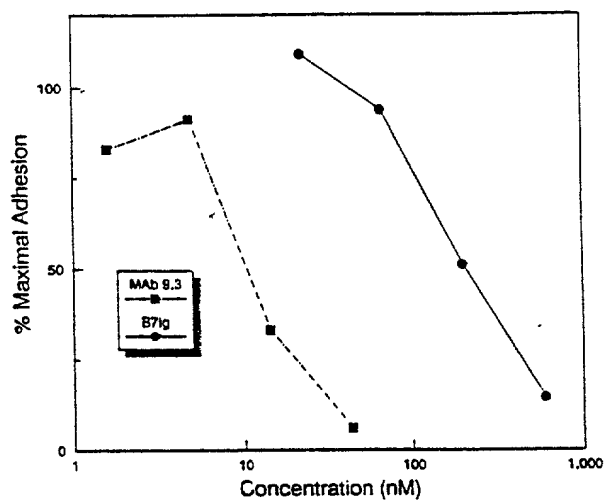


FIGURE 16

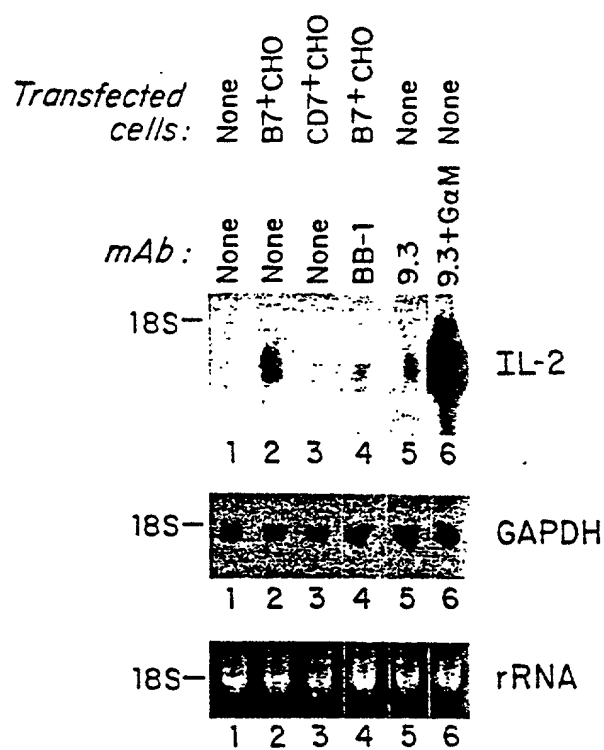


FIGURE 17

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION

As the below-named inventors, we hereby declare that:

Our residences, post office addresses and citizenship are as stated below next to our names.

We believe that we are original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled: LIGAND FOR CD28 RECEPTOR ON B CELLS AND METHODS, the specification of which:

_____ is attached hereto

XX was filed on June 27, 1991.

Application Serial Number: 07/722,101.

and was amended on (or amended through) _____
(if applicable)

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Sec. 1.56(a).

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

We hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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Inventor's Signature: 

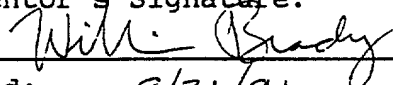
Dated: 8-19-91

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